



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C07K 14/47, 7/04</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 97/21728</b> <b>(43) International Publication Date:</b> 19 June 1997 (19.06.97)
<b>(21) International Application Number:</b> PCT/SE96/01621 <b>(22) International Filing Date:</b> 9 December 1996 (09.12.96)  <b>(30) Priority Data:</b> 9504467-3           12 December 1995 (12.12.95)   SE 60/009,386        29 December 1995 (29.12.95)   US  <b>(71) Applicant (for all designated States except US):</b> KAROLIN- SKA INNOVATIONS AB [SE/SE]; Karolinska Institutet, S-171 77 Stockholm (SE).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> NORDSTEDT, Chris- ter [SE/SE]; Nora Torg 37, S-118 34 Danderyd (SE). NÄSLUND, Jan [SE/SE]; c/o Larsson, Banérgatan 55, S-115 53 Stockholm (SE). THYBERG, Johan [SE/SE]; Karlavägen 47B, S-114 49 Stockholm (SE). TJERNBERG, Lars, O. [SE/SE]; Pumpbrinken 1A, S-163 56 Spånga (SE). TERENIUS, Lars [SE/SE]; Kyrkogårdsgatan 27, S-753 12 Uppsala (SE).  <b>(74) Agent:</b> AWAPATENT AB; P.O. Box 45086, S-104 30 Stockholm (SE).		<b>(81) Designated States:</b> AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> PEPTIDE BINDING THE KLVFF-SEQUENCE OF AMYLOID $\beta$  <b>(57) Abstract</b> <p>The invention relates to compounds of formula (I) or (II), which are of interest especially for inhibition of polymerization of amyloid <math>\beta</math> peptide, as model substances for synthesis of amyloid <math>\beta</math> peptide-ligands, as tools for the identification of other organic compounds with similar functional properties and/or as ligands for detection of amyloid deposits using e.g. positron emission tomography (PET). Formula (II) is: <math>R_1 - A' - Y' - Leu - X' - Z' - B' - R_2</math>, in which <math>X'</math> means any group or amino acid imparting to the compound according to formula (I) the ability to bind to the KLVFF-sequence in amyloid <math>\beta</math> peptide, or two amino acids imparting the same ability, but with the proviso that one is not proline; <math>Y'</math> means any amino acid; <math>Z'</math> means any non-acidic amino acid; <math>A'</math> means a direct bond or an <math>\alpha</math>-amino acid bonded at the carboxyl terminal of the <math>\alpha</math>-carboxy group or a di-, tri-, tetra- or pentapeptide bonded at the carboxyl terminal of the <math>\alpha</math>-carboxy group; <math>B'</math> means a direct bond or an <math>\alpha</math>-amino acid bonded at the <math>\alpha</math>-nitrogen or a di-, tri-, tetra- or pentapeptide bonded at the <math>\alpha</math>-nitrogen of the N-terminal <math>\alpha</math>-amino acid; <math>R_1</math> is H or <math>-CO-R_3</math> bonded at the <math>\alpha</math>-aminogroup of <math>A'</math>; <math>R_2</math> is H, <math>-OR_4</math> or <math>NR_5R_6</math>, all bonded to the <math>\alpha</math>-carboxyl group of the <math>\alpha</math>-carboxyterminal of <math>B'</math>; <math>R_3</math> and <math>R_4</math> are straight or branched carbon chain of 1-4 carbon atoms; <math>R_5</math> and <math>R_6</math> are independently H, alkyl, cycloalkyl, aryl or substituted aryl or together are <math>-(CH_2)_n-</math>, where n is 4-5; and <math>R_1</math> and <math>R_2</math> together can form a hydrocarbon ring or heterocyclic ring; all <math>\alpha</math>-amino acids being either D- or L-isomers.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam



## PEPTIDE BINDING THE KLVFF-SEQUENCE OF AMYLOID- $\beta$

### Introduction

5       The present invention relates to compounds,  
which are of special interest by their ability to  
bind to the KLVFF-sequence in the peptide amyloid  $\beta$   
and to inhibit polymerization of the amyloid  $\beta$   
peptide. The compounds according to the invention are  
10 e.g. useful as medicaments and as tools for identifi-  
cation of substances to be used in the treatment or  
prevention of amyloidosis.

### Background of the invention

15       Amyloidosis is a condition which is  
characterized by the deposition of amyloid in organs  
or tissues of the human or animal body, either as a  
primary disease of unknown cause or secondary to  
chronic disease, such as tuberculosis or osteomyeli-  
20 tis. In addition, it has also been found that the  
pre-eminent neuropathological feature of Alzheimer's  
disease (AD), a chronic condition of brain atrophy,  
is the deposition of amyloid in the brain parenchyma  
and cerebrovasculature (D.J. Selkoe, *Neuron* 6,  
25 487-498 (1991); D.J. Selkoe, *Annu. Rev. Cell Biol.*  
10, 373-403 (1994)).

      The basic component of such amyloid is a peptide  
termed amyloid  $\beta$ , or A $\beta$  (G.C. Glenner, C.W. Wong,  
*Biochem. Biophys. Res. Commun.* 120, 885-890 (1984)).  
30 It is a 40 to 42 amino acids long proteolytic frag-  
ment of the Alzheimer amyloid precursor protein  
(APP), a protein expressed in most tissues (J. Kang,  
*et al.*, *Nature* 325, 733-736 (1987)). Genetic and  
neuropathological studies provide strong evidence for  
35 a central role of A $\beta$  in the pathogenesis of AD, but  
the pathophysiological consequences of the amyloid

deposition are still unclear. However, it has been suggested that A $\beta$  polymers and amyloid are toxic to neurons, either directly or indirectly, and hence cause neurodegeneration (C. Behl, J. B. Davis, R. Lesley, D. Schubert, *Cell* 77, 817-827 (1994); D.T. Loo, et al., *ibid* 90, 7951-7955 (1995)).

The amyloid associated with Alzheimer's disease (AD) consists of thin fibrils of polymerized A $\beta$ . A rational pharmacological approach for the prevention of amyloidogenesis would therefore be to use drugs that specifically interfere with A $\beta$ -A $\beta$  interaction and polymerization. Previous studies showed that A $\beta$  polymerization *in vivo* and *in vitro* is a highly specific process, which probably involves an interaction between binding sequences in the A $\beta$  peptide (J. Näslund, et al., *Proc. Natl. Acad. Sci. USA* 91, 8378-8382 (1994); J. Näslund, et al., *Biochem. Biophys. Res. Commun.* 204, 780-787 (1994)).

Wood et al (S.J. Wood, R. Wetzel, J.D. Martin, M.R. Hurle, *Biochemistry* 34, 724-730 (1995)) suggest that amino acid residues within or close to A $\beta$ -16-20 are important for the adoption of the correct  $\beta$ -pleated sheet structure of A $\beta$  and show that amino acids 17-23 in the amyloid  $\beta$  peptide (A $\beta$ ) are essential for fibril formation and probably make up the  $\beta$ -sheet core of the fibrils. In addition, Wood et al. have investigated the ability of their peptides to form amyloid fibrils in a solution containing solely the mutated or the wild-type peptide. However, no method or principle which makes it possible to inhibit A $\beta$  of wild type from forming amyloid fibrils is devised and no use of the peptides as medicaments is suggested.

WO 95/08999 relates to amelioration of amnesia in Alzheimer's disease caused by deposition of amyloid  $\beta$  protein. Three peptides are disclosed, which overcome the amnestic effects of  $\beta$ -12-28, a peptide

homologous to A $\beta$ . In addition, WO 95/08999 describes the screening of several other peptides, which were neither significantly amnestic nor memory enhancing, of which one is KLVFF, SEQ. NO. 15 of the sequence  
5 listing therein.

In EP 0 584 452, novel amyloid precursor proteins and the sequences thereof are disclosed. Peptide sequences that comprise KLVFF are revealed. However, neither binding to amyloid  $\beta$  peptide nor any  
10 inhibition of the polymerization thereof is suggested.

#### Summary of the invention

Thus, the polymerization of the amyloid  $\beta$  peptide (A $\beta$ ) into amyloid fibrils is a critical step in  
15 the pathogenesis of Alzheimer's disease.

*In vitro* and *in vivo* studies of A $\beta$  have shown that the A $\beta$  molecules interact with a high degree of specificity during polymerization and fibril formation. It was assumed that ligands which bind to  
20 recognition sequences would be capable of inhibiting A $\beta$  polymerization and possibly also dissolve pre-formed A $\beta$  polymers *in situ*. The strategy in finding such A $\beta$  ligands was to identify critical binding regions in A $\beta$  and, based on their sequences, develop a  
25 compound capable of blocking the A $\beta$ -A $\beta$  binding.

According to the invention, it was hypothesized that compounds capable of binding to regions in the A $\beta$ -molecule critical for its polymerization might  
30 inhibit amyloid fibril formation, as described in more detail below.

According to the invention, it has now been found that the Lys-Leu-Val-Phe-Phe (KLVFF) sequence in A $\beta$  is necessary for polymerization to occur. Peptides incorporating this sequence bind to A $\beta$  and are  
35 capable of blocking the fibril formation of A $\beta$ -1-40 and are therefore potentially useful as drugs.

In addition, compounds have been found, which

- 1) are capable of binding to full-length A $\beta$ ,
- 2) are capable of blocking A $\beta$  fibril formation and
- 3) do not form fibrils by themselves.

5 In addition, it has also been found that alanine-substituted A $\beta$ -1-28 (Ala at position 16,17,20), in contrast to wild-type A $\beta$ -1-28, does not form fibrils.

10 Thus, it was concluded that the Lys-Leu-Val-Phe-Phe (16-20) motif serves as a structural basis for the development of peptide and non-peptide agents aimed at inhibiting amyloidogenesis *in vivo*. This is a novel finding and the compounds are of utmost interest as being useful as drugs for Alzheimer's

15 disease.

Further, the findings according to the invention are even more surprising on the basis of what was concluded from WO 95/08999 mentioned above. In WO 95/08999, it was concluded that KLVFF is not a poten-

20 tial candidate for the development of substances that can antagonize binding of A $\beta$  and thus attenuate symptoms and progression of AD. Even though the teaching of said WO publication indicates the opposite, according to the present invention, it has now been

25 found that KLVFF on the contrary is most useful for the development of new compounds defined by Formula (I) and (II) below.

#### Detailed description of the invention

30 The present invention relates to compounds which are able to bind to the Lys-Leu-Val-Phe-Phe-sequence, or KLVFF-sequence, in the peptide amyloid  $\beta$ . More specifically, the compounds according to the invention are defined by their formula (I):

35  $R_1 - A' - Y' - \text{Leu} - X' - Z' - B' - R_2$  (I)

in which

X' means any group or amino acid imparting to the compound of formula (I) the ability to bind to the KLVFF-sequence in amyloid  $\beta$  peptide, or two amino acids imparting the same ability, but with the proviso that one is

5 not proline;

Y' means any amino acid;

Z' means any non-acidic amino acid;

A' means a direct bond or an  $\alpha$ -amino acid bonded at the carboxyl terminal of the  $\alpha$ -carboxy group or a di-, tri-,  
10 tetra- or pentapeptide bonded at the carboxyl terminal of the  $\alpha$ -carboxy group;

B' means a direct bond or an  $\alpha$ -amino acid bonded at the  $\alpha$ -nitrogen or a di-, tri-, tetra- or pentapeptide bonded at the  $\alpha$ -nitrogen of the N-terminal  $\alpha$ -amino acid;

15 R<sub>1</sub> is H or -CO-R<sub>3</sub> bonded at the  $\alpha$ -amino group of A';

R<sub>2</sub> is H, -OR<sub>4</sub> or NR<sub>5</sub>R<sub>6</sub>, all bonded to the  $\alpha$ -carboxyl group of the  $\alpha$ -carboxyterminal of B';

R<sub>3</sub> is a straight or branched carbon chain of 1-4 carbon atoms;

20 R<sub>4</sub> is a straight or branched carbon chain of 1-4 carbon atoms;

R<sub>5</sub> and R<sub>6</sub> independently are H, alkyl, cycloalkyl, aryl or substituted aryl or together are -(CH<sub>2</sub>)<sub>n</sub>-, where n is 4-5;

R<sub>1</sub> and R<sub>2</sub> together can form a hydrocarbon ring or  
25 heterocyclic ring; and

all the  $\alpha$ -amino acids can be either D- or L-isomers; with the proviso that (I) is not Lys-Leu-Val-Phe-Phe.

With alkyl is preferably meant a chain of 4 or less carbon atoms, e.g. methyl, ethyl, propyl or

30 butyl.

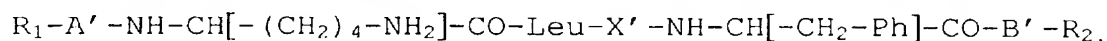
With cykloalkyl is preferably meant a ring of 3, 4, 5 or 6 carbon atoms.

Aryl preferably means a phenyl group, which can be substituted, preferably by a methyl, ethyl, propyl  
35 or butyl group, an amino or a methoxy, ethoxy, propoxy or butoxy group.

In a preferred embodiment of the present invention, the compound exhibits an ability to inhibit polymerization of amyloid  $\beta$  peptide.

In one embodiment of the invention, all the amino acids of the compound are D-isomers.

In one embodiment of the invention, Y' is Lys, and in a particular embodiment of the invention, Z' is Phe, resulting in a compound of the following formula:



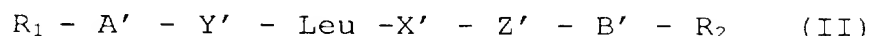
In an alternative embodiment of the invention, Y' is Phe.

Preferred are compounds, wherein X' is Val-Val.

In one embodiment of the present aspect of the invention, R<sub>1</sub> is acetyl.

In an alternative embodiment of the invention, R<sub>1</sub> is H. According to another embodiment, R<sub>2</sub> is H. Alternatively, R<sub>1</sub> and R<sub>2</sub> are both H.

A second aspect of the present invention is the use of a compound of formula:



in which

X' means any group or amino acid imparting to the compound of formula (II) the ability to bind to the KLVFF-sequence in amyloid  $\beta$  peptide, or two amino acids imparting the same ability, but with the proviso that one is not proline;

Y' means any amino acid;

Z' means any non-acidic amino acid;

A' means a direct bond or an  $\alpha$ -amino acid bonded at the carboxyl terminal of the  $\alpha$ -carboxy group or a di-, tri-, tetra- or pentapeptide bonded at the carboxyl terminal of the  $\alpha$ -carboxy group;

B' means a direct bond or an  $\alpha$ -amino acid bonded at the  $\alpha$ -nitrogen or a di-, tri-, tetra- or pentapeptide bonded at the  $\alpha$ -nitrogen of the N-terminal  $\alpha$ -amino acid;

R<sub>1</sub> is H or -CO-R<sub>3</sub> bonded at the α-amino group of A';  
R<sub>2</sub> is H, -OR<sub>4</sub> or NR<sub>5</sub>R<sub>6</sub>, all bonded to the α-carboxyl group  
of the α-carboxyterminal of B';

5 R<sub>3</sub> is a straight or branched carbon chain of 1-4 carbon  
atoms;

R<sub>4</sub> is a straight or branched carbon chain of 1-4 carbon  
atoms;

R<sub>5</sub> and R<sub>6</sub> independently are H, alkyl, cycloalkyl, aryl or  
substituted aryl or together are -(CH<sub>2</sub>)<sub>n</sub>- where n is 4-5;

10 R<sub>1</sub> and R<sub>2</sub> can together form a hydrocarbon ring or  
heterocyclic ring; and

all the α-amino acids can be either D- or L-isomers;  
for inhibition of polymerization of amyloid β pep-  
tide, as a model substance for synthesis of amyloid β  
15 peptide-ligands for inhibition of polymerization of  
amyloid β peptide, as a tool for the identification  
of other organic compounds with similar functional  
properties or as ligand for detection of amyloid de-  
posits using e.g. positron emission tomography (PET).

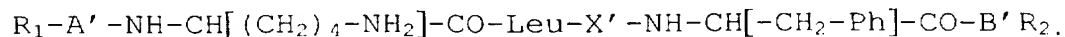
20 With alkyl is preferably meant a chain of 4 or  
less carbon atoms, e.g. methyl, ethyl, propyl or  
butyl.

With cykloalkyl is preferably meant a ring of 3,  
4, 5 or 6 carbon atoms.

25 Aryl preferably means a phenyl group, which can  
be substituted, preferably by a methyl, ethyl, propyl  
or butyl group, an amino or a methoxy, ethoxy,  
propoxy or butoxy group.

In one embodiment of this second aspect of the  
30 invention, a compound is used, wherein all the amino  
acids are D-isomers.

In a particular embodiment of this aspect of the  
invention, Y' is Lys. A particular embodiment is when  
Z' is Phe, resulting in a compound of the following  
35 formula:



In an alternative embodiment, a compound is used, wherein Y' is Phe while Z' is any non-acidic amino acid.

In a preferred embodiment of this aspect of the invention, a compound is used, wherein X' is Val-Val.

In one embodiment of the use according to the invention, R<sub>1</sub> is acetyl. Alternatively, R<sub>1</sub> and/or R<sub>2</sub> are H.

Yet another aspect of the present invention is a compound according to the invention for use as a medicament.

Also claimed is the use of a compound, preferably of the formula (I) or (II), which is able to bind to the KLVFF-sequence in amyloid  $\beta$  peptide and which has the ability to inhibit polymerization of amyloid  $\beta$  peptide, for the manufacture of a medicament for the treatment or prevention of amyloidosis, especially in the treatment or prevention of Alzheimer's disease associated with amyloidosis, for the treatment or prevention of demens in patients with Down's syndrome, for the treatment or prevention of Hereditary cerebral hemorrhage with amyloidosis (Dutch type) or for the prevention of fibril formation of human amyloid protein.

Further, a last aspect of the present invention is a composition comprising a compound according to formula II and optionally a ligand capable of binding or interacting with the compound according to formula II and a carrier.

Said composition can e.g. be adapted for injection in a liquid carrier or for oral administration in a tablet or capsule.

Carriers are known for persons skilled in the art.

For clarification the following definitions are given:



K is lysine (Lys), L is leucine (Leu), V is valine (Val), F is phenylalanine (Phe), A is alanine (Ala) and E is glutamic acid (Glu).

As used herein, "any group giving the compound according to formula (I) the ability to bind to the KLVFF-sequence in the amyloid  $\beta$  peptide" means that this group gives the compound a structure, which can fulfil the requirements given in claim 1.

The hydrocarbon ring or heterocyclic ring has preferably 4-6 atoms, preferably C, N and S.

#### Description of the figures

Fig. 1 A and B. A $\beta$ -amyloid polymerization.

Fig. 2A. Ten-mers corresponding to consecutive sequences of A $\beta$ -1-40. Radioactivity bound to the filter was detected by autoradiography and quantified by densitometry.

Fig. 2B. EVHHQ**KLVFF** and N and C-terminal truncated fragments were synthesized and analyzed for affinity to  $^{125}\text{I}$ -labelled A $\beta$ -1-40.

Fig. 2C. Each amino acid residue in KLVFF was systematically replaced with Ala and analyzed for affinity to  $^{125}\text{I}$ -labelled A $\beta$ -1-40.

Fig 2D. Sensorgram from surface plasmon resonance spectroscopy (BIAcore 2000).

Fig. 3 A and B. Content of non-aggregated peptide in the supernatants from incubations of wild-type and Ala-substituted A $\beta$ -1-28 as analyzed by HPLC.

#### 30 EXPERIMENTAL

##### Example 1

a) Ten-mers corresponding to consecutive sequences of A $\beta$ -1-40 were synthesized on a filter matrix using the SPOT-technique (the peptides were synthesized essentially as described by Frank [R. Frank, *Tetrahedron* 42, 9217-9232 (1992)]. Briefly, a spacer corresponding to 2 molecules of  $\beta$ -alanine was coupled to cellu-

lose membranes (Whatman 1Chr). The peptides were synthesized on these derivatized membranes using Fmoc protected and pentafluorophenyl-activated amino acids (AMS Biotechnology) dissolved in N-methylpyrrolidone.

5 Coupling efficiency was monitored using bromphenol blue.). We synthesized the thirty-one possible 10-mers of the A $\beta$ -1-40. Peptide no. 1 corresponds to amino acids 1-10, peptide no. 2 to amino acids 2-11 etc. The filter-bound peptides were incubated with

10 radioactive A $\beta$ -1-40. Following washing of the filter in high-salt buffer, bound radioactivity was estimated by autoradiography and densitometry. Following blocking with 0.05% Tween-20 in Tris-buffered saline (TBS), the filter was incubated in the presence of 20

15  $\mu$ M  $^{125}$ I-labelled A $\beta$ -1-40 at 20°C for 12 h in TBS, pH 7.3, supplemented with 1% bovine serum albumin. The filter was then washed repeatedly in the same buffer containing 0.5 M NaCl and dried. Radioactivity bound to the filter was visualized by autoradiography and

20 quantitated using a densitometer.

b) Peptide no. 11 (EVHHQ**KLVFF**) and indicated N- and C-terminal truncated fragments were synthesized using the same technique as described above and analyzed for affinity to  $^{125}$ I-labelled A $\beta$ -1-40.

25 c) Sensorgram from BIAcore 2000. A $\beta$ -1-40, at three different concentrations in running buffer, pH 7.4. A $\beta$ -1-40 was injected during 10 minutes over a sensor-chip derivatized with the peptide KLVFF- $\beta$ A- $\beta$ A-C.

d) Each amino acid residue in KLVFF was systematically replaced with A and analyzed for affinity to

30  $^{125}$ I-labelled A $\beta$ -1-40. Non-specific interactions have been compensated for by subtracting the signal from a surface derivatized with C alone.

### 35 Results

a) The measured binding should be interpreted as semiquantative, since the coupling efficiency and

therefore the amount of peptide per spot may vary. A region located in the central part of A $\beta$  (A $\beta$ -9-18 to A $\beta$ -13-22) displayed prominent binding of radioactive A $\beta$ -1-40. Another binding region was the hydrophobic C-terminus of the molecule (D. Burdick, et al, *J. Biol. Chem.* 267, 546-554 (1992)) but binding here was considerably weaker (Fig 2A).

b) Being located in the centre of the binding region, peptide no. 11 (corresponding to A $\beta$ -11 - 20) was selected for further studies. This peptide, as well as N- and C-terminal fragments thereof, were synthesized using the same technique as described previously. The shortest peptide still displaying high A $\beta$  binding capacity had the sequence KLVFF, corresponding to amino acids 16-20 of A $\beta$  (Fig. 2B). By systematically substituting the amino acid residues in the KLVFF sequence with alanine, we found that the first, second and fifth residues (i.e. KLXXF) were critical for binding (Fig. 2C).

c) The interaction between soluble A $\beta$ -1-40 and immobilized KLVFF was monitored in real-time (Fig. 2D) using surface plasmon resonance spectroscopy (BIAcore, Pharmacia) (BIAcore 2000 (Pharmacia Biosensor AB, Sweden) was used for real-time studies based on surface plasmon resonance spectroscopy. The peptide was immobilized using thiol coupling. The running buffer consisted of 10 mM HEPES, 0.15 M NaCl, 3.4 mM EDTA and 0.05% surfactant P20 as described. [U. Jansson, M. Malmqvist, *Adv. Biosens.* 2, 291-336 (1992)]). The binding was not saturable, indicating that A $\beta$ -1-40 bound to immobilized KLVFF could interact with other A $\beta$ -1-40 molecules in a polymerization reaction.

d) AA served as linker between the active peptide and the chip (upper trace) and cysteine alone, indicating non-specific binding, (C) as control (lower

trace). Arrows indicate start and stop of injection (Fig. 2D).

### Example 2

5 To investigate if the KLXXF motif was required for A $\beta$  polymerization, we synthesized A $\beta$ -1-28, a well-studied A $\beta$  fragment that readily forms amyloid fibrils (D.A. Kirschner, *et al.*, *Proc. Natl. Acad. Sci. USA* 84, 6953-6957 (1987); C.J. Barrow, M.G. Zagorski, *Science* 253, 179-82 (1991); C. Nordstedt, *et al.*, *J. Biol. Chem.* 269, 30773-30776 (1994)) and mutated A $\beta$ -1-28 where the KLVFF sequence was substituted with **AAVFA** (A $\beta$ -1-28**AAVFA**).

15 A $\beta$ -1-28 (Fig. 3 A) and A $\beta$ -1-28**AAVFA** (Fig. 3 B) were incubated at 200  $\mu$ M in TBS for 24 h at 37°C in a shaking water bath. After incubation, the tubes were centrifuged at 20,000g for 20 min and the content of non-aggregated peptide in the supernatants (Fig. 3 A, B) was analyzed using an established C4 RPLC system (12) whereas the aggregated peptides in the pellets were analyzed by electron microscopy after adsorption to formvar-coated grids and negative staining with 2% uranyl acetate in water.

### Results

25 Following incubation at a concentration of 200  $\mu$ M for 24 h at 37°C, A $\beta$  -1-28 aggregated (Fig. 3A) and formed large fibril bundles, whereas A $\beta$ -1-28**AAVFA** almost completely failed to aggregate (Fig. 3B) and only formed a few dispersed fibrils.

### Example 3

35 A $\beta$ -1-40 was incubated at 100  $\mu$ M in TBS for 48 h at 37°C in a shaking water bath, either alone or together with 100  $\mu$ M AcKLVFFNH<sub>2</sub>. The polymerized material was adsorbed to formvar-coated grids and negatively stained with 2% uranyl acetate in water.

### Results

Incubation of synthetic A $\beta$ -1-40 at 100  $\mu$ M for 48 h at 37°C in a physiological buffer led to polymeri-  
5 zation and formation of amyloid fibrils arranged in parallel in densely packed bundles, as previously shown (C. Nordstedt, et al., *J. Biol. Chem.* 269, 30773-30776 (1994)). When A $\beta$ -1-40 was coincubated with AcQ**KLVFF**NH<sub>2</sub> at equimolar concentrations, this  
10 type of fibrils did not form. Instead, only a few occasional fibrils embedded in a diffuse background of small rod-like aggregates, similar to those formed by AcQ**KLVFF**NH<sub>2</sub> itself, could be detected.

### Example 4

The peptides were synthesized essentially as described by Frank (Frank R, 1992, Tetrahedron 42:9217-9232). Briefly, a spacer corresponding to 2 molecules of  $\beta$ -alanine was coupled to cellulose mem-  
20 branes (Whatman XX). The peptides were synthesized on these derivatized membranes using Fmoc protected and pentafluorophenyl-activated amino acids (AMS biotechnology) dissolved in N-methylpyrrolidone. Coupling efficiency was monitored using bromphenol blue.

25

### Results

The KLXXF motif in the A $\beta$  molecule is not only critical for polymerization and fibril formation. During non-amyloidogenic processing of APP, the mole-  
30 cule is cleaved between amino acid residues K<sup>16</sup> and L<sup>17</sup> (F.S. Esch, et al., *Science* 248, 1122-1124 (1990)), leading to the formation of a fragment of A $\beta$  termed p3 and corresponding to A $\beta$ -17-40 or A $\beta$ -17-42 (C. Haass, A.Y. Hung, M.G. Schlossmacher, D.B. Teplow, D. J. Selkoe, *J. Biol. Chem.* 268, 3021-3024 (1993)). Through this metabolic pathway the present binding sequence is disrupted. This may explain why

p3 is not capable of forming amyloid *in vitro* or *in vivo* (J. Näslund, et al., *Proc. Natl. Acad. Sci. USA* 91, 8378-8382 (1994); J. Näslund, et al., *Biochem. Biophys. Res. Commun.* 204, 780-787 (1994)). The  
5 KLXXF motif is highly sequence specific. The most apparent example of this is the finding that substitution of a single amino acid leads to virtually complete loss of A $\beta$  binding capacity.

10 Example 5

In an additional series of experiments, it was demonstrated that KLVFF binds stereo specifically to the homologous sequence in A $\beta$  (i.e. A $\beta$ -16-20). By screening combinatorial pentapeptide libraries exclusively composed  
15 of D-amino acids (lowercase) with labelled KLVFF, several ligands with a motif containing phenylalanine (f) in the second and leucine (l) in the third position were identified (e.g. lflrr). By using a short peptide in the screening, known to bind to a region in A $\beta$  critical for its  
20 polymerization (i.e. KLVFF), the risk of identifying D-pentapeptides that interact with nonrelevant regions in A $\beta$  (N- or C-terminal to A $\beta$ -16-20) was eliminated. Like KLVFF, the D-amino acid ligands were found not only to bind to A $\beta$  but also to inhibit amyloid fibril formation.  
25 Since peptides built up of D-amino acids are resistant to proteolytic degradation, these ligands may be beneficial for inhibition of amyloidogenesis *in vivo*. The results further indicate that KLVFF will be useful in the identification of small organic molecules (e.g. by screening of  
30 substance libraries) with the ability to bind to A $\beta$  in this relevant region and inhibit amyloid fibril formation (candidate drugs for the treatment of Alzheimer disease and other related amyloidoses).

Discussion and conclusion

Previous studies of putative inhibitors of amyloid fibril formation showed that cyclodextrins (P. Camillieri, N.J. Haskind, D.R. Howlett, *FEBS Lett.* 5 341, 256-258 (1994)) and Congo red (A. Lorenzo, B. Yankner, *Proc. Natl. Acad. Sci. USA* 91, 12243-12247 (1994)) may have such properties. The usefulness of these molecules as lead or model substances in development of anti-Alzheimer amyloid drugs is, however, 10 compromised by their relative lack of specificity. Cyclodextrins have primarily been used to increase the solubility of a wide range of lipophilic drugs and it is unlikely that they would display any specificity for A $\beta$  *in vivo*. Congo red, which is used in 15 histochemistry to detect amyloid, binds to a wide array of non-A $\beta$  amyloids as well as to other proteins with a high content of  $\beta$ -pleated sheet structures (W.G. Turnell, J.T. Finch, *J. Mol. Biol.* 227, 1205-1223 (1992)).

20 Due to the extreme insolubility of amyloid, strong chaotropic agents or potent organic solvents are required for its dissolution (C.L. Masters, et al., *Proc. Natl. Acad. Sci. USA* 82, 4245-4249 (1985)), the concept of dissolving amyloid deposits 25 *in situ* under physiological conditions may seem futile. However, the bulk of the individual molecules in amyloid are probably not joined by covalent bonds and the deposition of A $\beta$  into amyloid is, at least at some stages, a dynamic and reversible process (J.E. Maggio, et al., *ibid.*, 89, 5462-5466 30 (1992)). Hence, a molecule capable of binding to a site in the A $\beta$  molecule that is critical for fibril formation with an affinity higher than native A $\beta$  should have reasonable chances to inhibit amyloid 35 growth and maybe also specifically dissolve amyloid fibrils.

In conclusion, we have identified an A $\beta$  sequence, KLVFF, which is required for amyloid fibril formation. The KLVFF peptide may serve as a model substance for the synthesis of non-peptide A $\beta$ -ligands that interfere with the polymerization of A $\beta$  molecules.

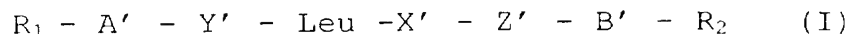
Previous studies suggested that amino acid residues within or close to A $\beta$ -16-20 are important for the adoption of the correct  $\beta$ -pleated sheet structure of A $\beta$  (18) and the proteolytic processing of its precursor (14).

We have now shown that this region harbors at least one binding sequence required for the polymerization of A $\beta$  into amyloid fibrils. It was also demonstrated that short peptides incorporating A $\beta$ -16-20 can function as ligands that bind to A $\beta$  and inhibit the formation of amyloid fibrils. Since these peptide ligands are relatively small, they are amenable for identification of other organic molecules with similar functional properties. Non-peptide homologues of KLVFF should be useful as pharmacological drugs for the treatment of Alzheimer's disease in the future.



CLAIMS

1. A compound having the formula



5

in which

X' means any group or amino acid imparting to the compound of formula (I) the ability to bind to the KLVFF-sequence in amyloid  $\beta$  peptide, or two amino acids imparting the same ability, but with the proviso that one is not proline;

Y' means any amino acid;

Z' means any non-acidic amino acid;

A' means a direct bond or an  $\alpha$ -amino acid bonded at the carboxyl terminal of the  $\alpha$ -carboxygroup or a di-, tri-, tetra- or pentapeptide bonded at the carboxyl terminal of the  $\alpha$ -carboxy group;

B' means a direct bond or an  $\alpha$ -amino acid bonded at the  $\alpha$ -nitrogen or a di-, tri-, tetra- or pentapeptide bonded at the  $\alpha$ -nitrogen of the N-terminal  $\alpha$ -amino acid;

R<sub>1</sub> is H or -CO-R<sub>3</sub> bonded at the  $\alpha$ -amino group of A';

R<sub>2</sub> is H, -OR<sub>4</sub> or NR<sub>5</sub>R<sub>6</sub>, all bound to the  $\alpha$ -carboxyl group of the  $\alpha$ -carboxyterminal of B';

R<sub>3</sub> is a straight or branched carbon chain of 1-4 carbon atoms;

R<sub>4</sub> is a straight or branched carbon chain of 1-4 carbon atoms;

R<sub>5</sub> and R<sub>6</sub> independently are H, alkyl, cycloalkyl, aryl or substituted aryl or together are -(CH<sub>2</sub>)<sub>n</sub>-, where n is 4-5;

R<sub>1</sub> and R<sub>2</sub> together can form a hydrocarbon ring or heterocyclic ring; and

all the  $\alpha$ -amino acids can be either D- or L-isomers; with the proviso that (I) is not Lys-Leu-Val-Phe-Phe.

2. A compound according to claim 1, which exhibits an ability to inhibit polymerization of amyloid  $\beta$  peptide.

3. A compound according to any one of claims 1-2, wherein all the amino acids are D-isomers.

4. A compound according to any one of the preceding claims, wherein Y' is Lys.

5 5. A compound according to claim 4, wherein Y' is Lys and Z' is Phe.

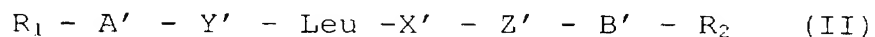
6. A compound according to any one of claims 1-3, wherein Y' is Phe.

7. A compound according to any one of the preceding claims, wherein X' is Val-Val.

8. A compound according to any one of the preceding claims, wherein R<sub>1</sub> is acetyl.

9. A compound according to any one of claims 1-8, wherein R<sub>1</sub> is H and/or R<sub>2</sub> is H.

15 10. Use of a compound of formula



in which

20 X' means any group or amino acid imparting to the compound of formula (II) the ability to bind to the KLVFF-sequence in the amyloid  $\beta$  peptide, or two amino acids imparting the same ability, but with the proviso that one is not proline;

25 Y' means any amino acid;

Z' means any non-acidic amino acid;

A' means a direct bond or an  $\alpha$ -amino acid bonded at the carboxyl terminal of the  $\alpha$ -carboxy group or a di-, tri-, tetra- or pentapeptide bonded at the carboxyl terminal of the  $\alpha$ -carboxy group;

B' means a direct bond or an  $\alpha$ -amino acid bonded at the  $\alpha$ -nitrogen or a di-, tri-, tetra- or pentapeptide bonded at the  $\alpha$ -nitrogen of the N-terminal  $\alpha$ -amino acid;

R<sub>1</sub> is H or -CO-R<sub>3</sub> bonded at the  $\alpha$ -amino group of A';

35 R<sub>2</sub> is H, -OR<sub>4</sub> or NR<sub>5</sub>R<sub>6</sub>, all bound to the  $\alpha$ -carboxyl group of the  $\alpha$ -carboxyterminal of B';

R<sub>3</sub> is a straight or branched carbon chain of 1-4 carbon atoms;

R<sub>4</sub> is a straight or branched carbon chain of 1-4 carbon atoms;

5 R<sub>5</sub> and R<sub>6</sub> independently are H, alkyl, cycloalkyl, aryl or substituted aryl or together are -(CH<sub>2</sub>)<sub>n</sub>-, where n is 4-5; R<sub>1</sub> and R<sub>2</sub> together can form a hydrocarbon ring or heterocyclic ring;

all the α-amino acids can be either D- or L-isomers;

10 for inhibition of polymerization of amyloid β peptide, as model substance for synthesis of amyloid β peptide-ligands for inhibition of polymerization of amyloid β peptide, as a tool for the identification of other organic compounds with similar functional  
15 properties or as a ligand in PET (positron emission tomography).

11. Use according to claim 10, wherein all the amino acids of the compound are D-isomers.

12. Use according to any one of claims 10-11,  
20 wherein Y' is Lys.

13. Use according to claim 12, wherein Y' is Lys and Z' is Phe.

14. Use according to any one of claims 10-11, wherein Y' is Phe.

25 15. Use according to any one of claims 10-14, wherein X' is Val-Val.

16. Use according to any one of claims 10-15, wherein R<sub>1</sub> is acetyl.

17. Use according to any one of claims 10-15,  
30 wherein R<sub>1</sub> is H and/or R<sub>2</sub> is H.

18. A compound according to any one of claims 1-9 for use as a medicament.

19. Use of a compound according to any one of claims 1-9 for the manufacture of a medicament for  
35 the treatment or prevention of amyloidosis.

20. Use of a compound according to any one of claims 1-9 for the manufacture of a medicament for

the treatment or prevention of Alzheimer disease associated with amyloidosis.

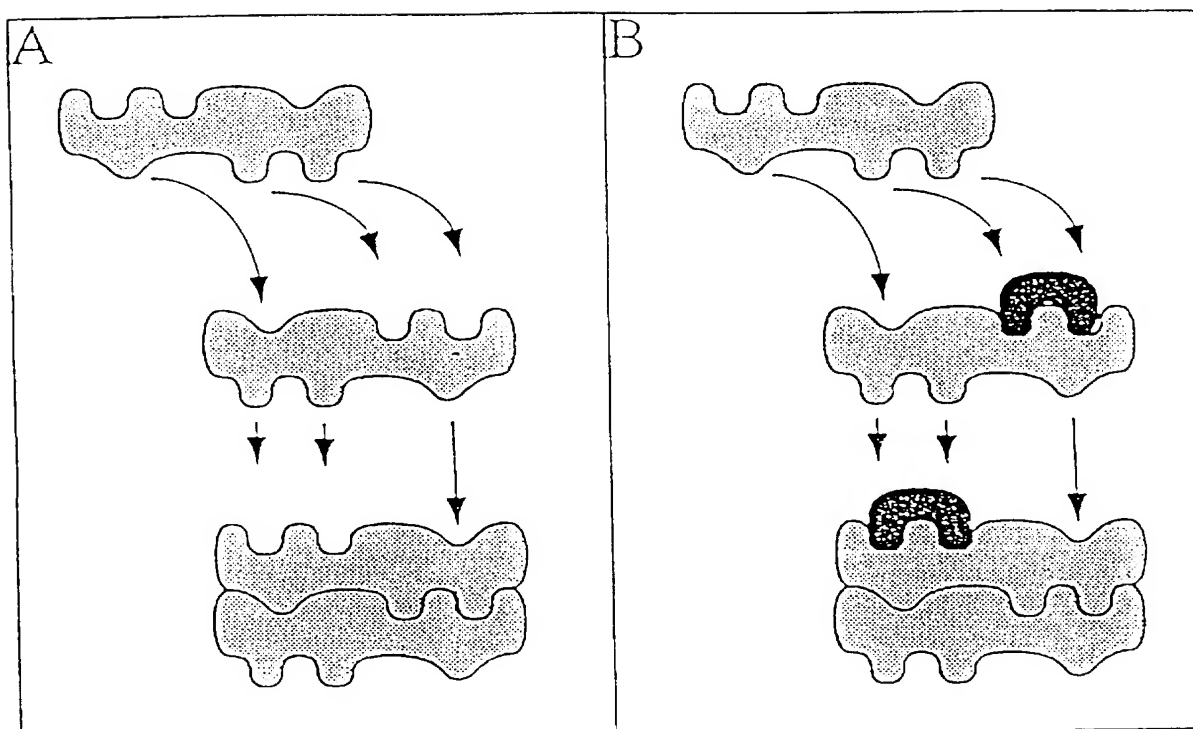
21. Use of a compound according to any one of claims 1-9 for the manufacture of a medicament for the treatment or prevention of demens in patients with Down's syndrome.

22. Use of a compound according to any one of claims 1-9 for the manufacture of a medicament for the treatment or prevention of Hereditary cerebral hemorrhage with amyloidosis (Dutch type).

23. Use of a compound according to any one of claims 1-9 for the manufacture of a medicament for the prevention of fibril formation of human amyloid protein.

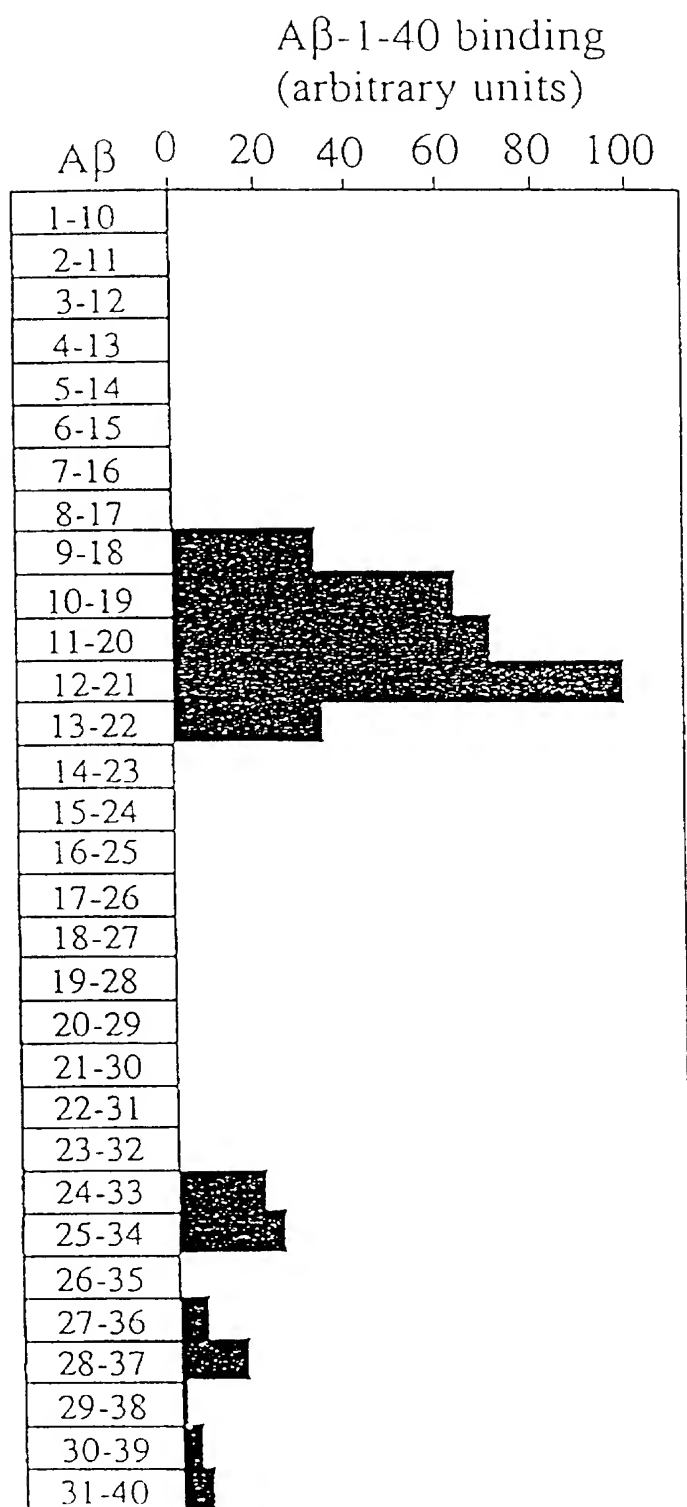
24. A composition comprising a compound according to any one of claims 1-9 and optionally a ligand capable of binding or interacting with the compound according to formula I and a carrier.

25. A composition according to claim 24, which is adapted for injection or oral administration.



2/6

Fig. 2 A.



Peptide  
sequence

A $\beta$ -1-40 binding  
(arbitrary units)

0 25 50 75 100

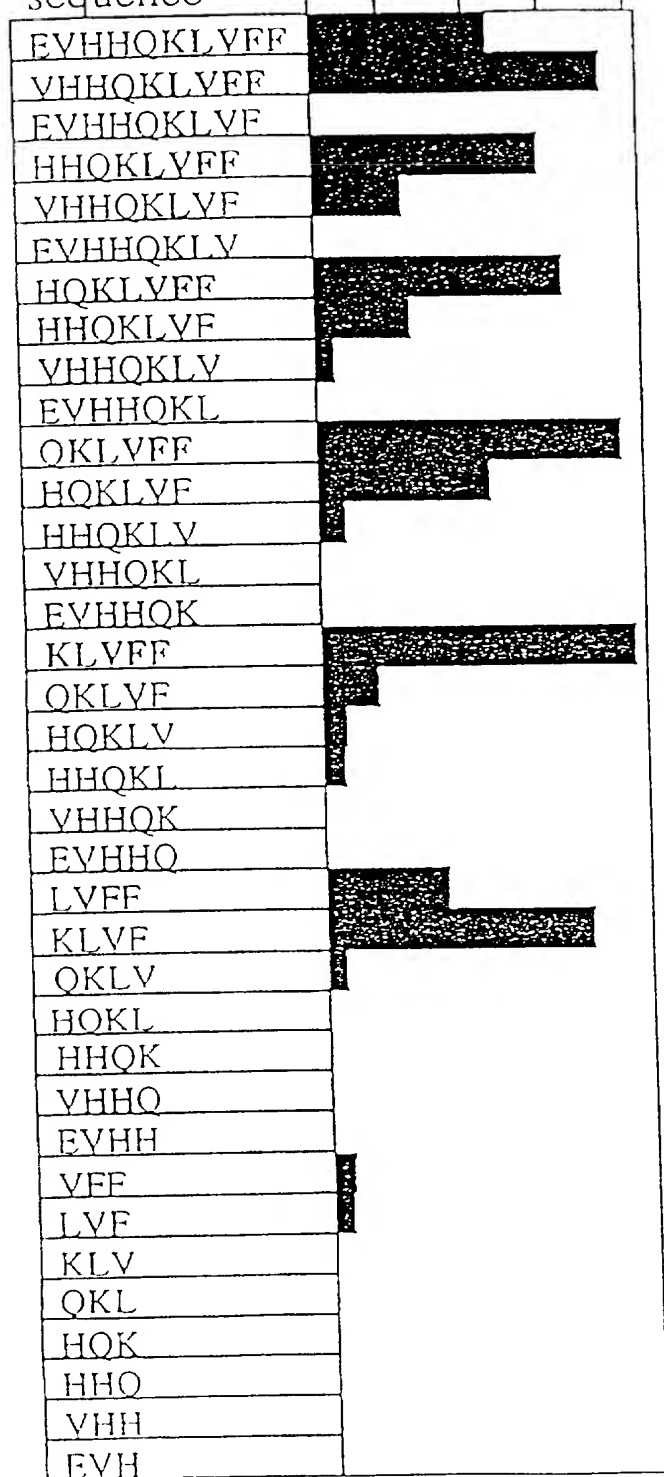


Fig. 2 B.

Fig. 2 C.

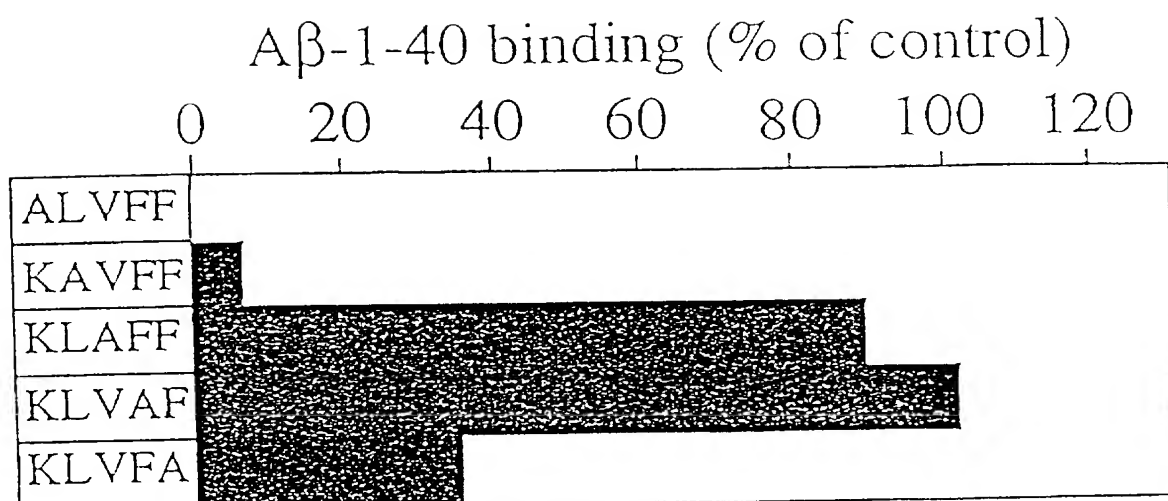




Fig. 2 D.

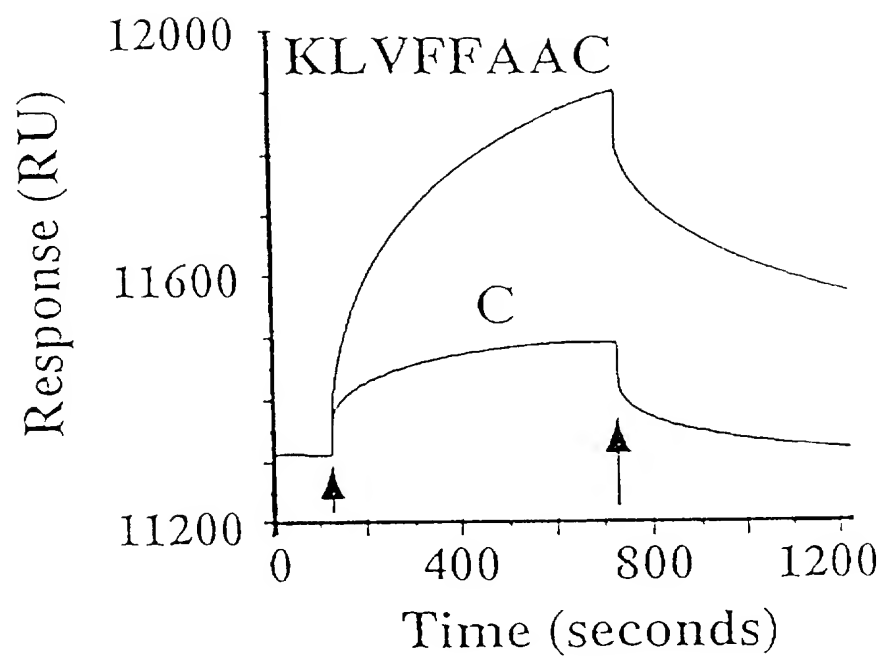
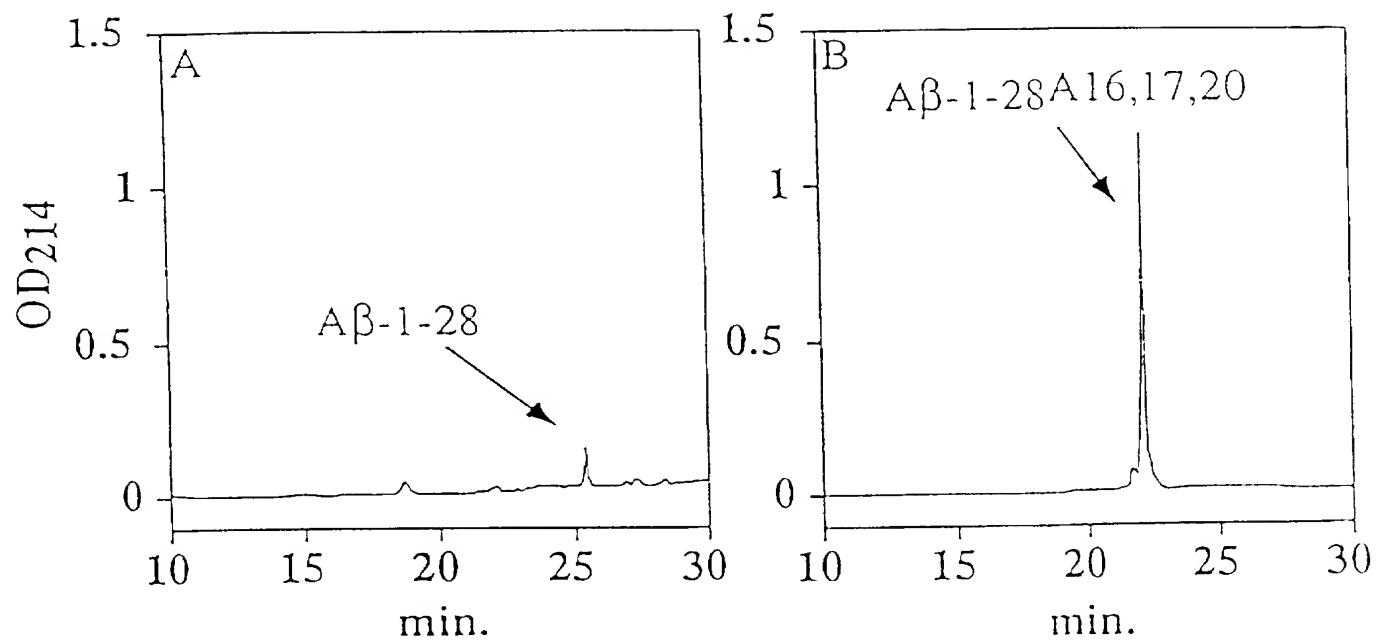


Fig. 3 A-B.



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 96/01621

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/47, C07K 7/04

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, WPI, CA, REG, EPOQUE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9508999 A1 (CITY OF HOPE), 6 April 1995 (06.04.95), page 3, line 26 - line 36, page 8, line 27 - page 9, line 17, SEQ ID No 5, 14, 15 --	1-25
X	EP 0584452 A1 (AMERICAN CYANAMID COMPANY), 2 March 1994 (02.03.94), SEQ ID No 7, 25, 27 --	1-25
X	WO 9419692 A1 (THE GENERAL HOSPITAL CORPORATION), 1 Sept 1994 (01.09.94), SEQ ID No 11 --	1-3, 9-11, 17-25
P, X	WO 9634887 A2 (IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY & MEDICINE), 7 November 1996 (07.11.96), claims 10, 11, 13 --	1-6

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 March 1997

Date of mailing of the international search report

26 -03- 1997

Name and mailing address of the ISA/  
Swedish Patent Office  
Box 5055, S-102 42 STOCKHOLM  
Facsimile No. +46 8 666 02 86

Authorized officer

Patrick Andersson  
Telephone No. +46 8 782 25 00

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 96/01621

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9414836 A1 (CENTOCOR, INC.), 7 July 1994 (07.07.94), claim 15  -- -----	1-4,8-9

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

04/03/97

International application No.

PCT/SE 96/01621

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO-A1-	9508999	06/04/95	EP-A-	0670731	13/09/95
			US-A-	5470951	28/11/95
EP-A1-	0584452	02/03/94	AU-B-	667895	18/04/96
			AU-A-	3835893	04/11/93
			CA-A-	2095421	02/11/93
			JP-A-	7132094	23/05/95
			NZ-A-	247499	26/04/96
			ZA-A-	9303084	24/11/93
WO-A1-	9419692	01/09/94	US-A-	5578451	26/11/96
WO-A2-	9634887	07/11/96	AU-A-	5654096	21/11/96
			GB-D-	9509263	00/00/00
			GB-D-	9607505	00/00/00
WO-A1-	9414836	07/07/94	NONE		

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 6 :</b> <b>C07K 16/00, C12N 15/12, 15/13, 15/63,</b> <b>G01N 33/53, A61K 38/17, 39/395</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/02462</b> <b>(43) International Publication Date:</b> 22 January 1998 (22.01.98)
<b>(21) International Application Number:</b> PCT/EP97/03792 <b>(22) International Filing Date:</b> 16 July 1997 (16.07.97)  <b>(30) Priority Data:</b> 96111441.0 16 July 1996 (16.07.96) EP <b>(34) Countries for which the regional or international application was filed:</b> DE et al.  <b>(71) Applicant (for all designated States except US):</b> MORPHOSYS GESELLSCHAFT FÜR PROTEINOPTIMIERUNG MBH [DE/DE]; Am Klopferspitz 19, D-82152 Martinsried (DE).  <b>(71)(72) Applicant and Inventor:</b> PLÜCKTHUN, Andreas [DE/CH]; Möhrlistrasse 97, CH-8006 Zürich (CH).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> NIEBA, Lars [DE/CH]; Nordstrasse 304, CH-8037 Zürich (CH). HONEGGER, An- nemarie [CH/CH]; Murwiesenstrasse 32, CH-8057 Zürich (CH).  <b>(74) Agent:</b> VOSSIUS & PARTNER GBR; P.O. Box 86 07 67, D- 81634 München (DE).		<b>(81) Designated States:</b> CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> IMMUNOGLOBULIN SUPERFAMILY DOMAINS AND FRAGMENTS WITH INCREASED SOLUBILITY		
<b>(57) Abstract</b> <p>The present invention relates to the modification of immunoglobulin superfamily (IgSF) domains, IgSF fragments and fusion proteins thereof, especially to the modification of antibody derivatives, so as to improve their solubility, and hence the yield, and ease of handling. The inventors have found that this can be achieved by making the region which comprises the interface with domains adjoined to said IgSF domain in a larger fragment or a full IgSF protein, and which becomes exposed in the IgSF domain, more hydrophilic by modification. The present invention describes DNA sequences encoding modified IgSF domains or fragments and fusion proteins thereof, vectors and hosts containing these DNA sequences, IgSF domains or fragments or fusion proteins obtainable by expressing said DNA sequences in suitable expression systems, and a method for modifying IgSF domains, so as to improve their solubility, expressibility and ease of handling.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## **Immunoglobulin Superfamily Domains and Fragments with Increased Solubility**

### **Field of the Invention**

The present invention relates to the modification of immunoglobulin superfamily (IgSF) domains and derivatives thereof so as to increase their solubility, and hence the yield, and ease of handling.

### **Background to the Invention**

Small antibody fragments show exciting promise for use as therapeutic agents, diagnostic reagents, and for biochemical research. Thus, they are needed in large amounts, and the expression of antibody fragments, e.g. Fv, single-chain Fv (scFv), or Fab in the periplasm of *E. coli* (Skerra & Plückthun 1988; Better et al., 1988) is now used routinely in many laboratories. Expression yields vary widely, however, especially in the case of scFvs. While some fragments yield up to several mg of functional, soluble protein per litre and OD of culture broth in shake flask culture (Carter et al., 1992; Plückthun et al. 1996), other fragments may almost exclusively lead to insoluble material, often found in so-called inclusion bodies. Functional protein may be obtained from the latter in modest yields by a laborious and time-consuming refolding process. The factors influencing antibody expression levels are still only poorly understood. Folding efficiency and stability of the antibody fragments, protease lability and toxicity of the expressed proteins to the host cells often severely limit actual production levels, and several attempts have been tried to increase expression yields. For example, Knappik & Plückthun (1995) have identified key residues in the antibody framework which influence expression yields dramatically. Similarly, Ullrich et al. (1995) found that point mutations in the CDRs can increase the yields in periplasmic antibody fragment expression. Nevertheless, these strategies are only applicable to a few antibodies.

The observations by Knappik & Plückthun (1995) indicate that optimizing those parts of the antibody fragment which are not directly involved in antigen recognition can significantly



improve folding properties and production yields of recombinant Fv and scFv constructs. The causes for the improved expression behavior lie in the decreased aggregation behavior of these molecules. For other molecules, fragment stability and protease resistance may also be affected. The understanding of how specific sequence modifications change these properties is still very limited and currently under active investigation.

Difficulties in expressing and manipulating protein domains may arise because amino acids which are normally buried within the protein structure become exposed when only a portion of the whole molecule is expressed. Aggregation may occur through interaction of newly solvent-exposed hydrophobic residues originally forming the contact regions between adjacent domains. Leistler and Perham (1994) could show that a certain domain of glutathione reductase may be expressed separately from its neighboring domains, but the protein showed non-specific association *in vitro* forming multimeric protein species. The introduction of hydrophilic residues instead of exposed hydrophobic amino acids could decrease this aggregation tendency and thus stabilize this isolated domain. Both wild type and modified domains were exclusively found in inclusion bodies and had to be refolded. Although *in vitro* experiments contributed a lot to define various intermolecular interactions, which drive folding processes, they are only of limited value in predicting the folding behaviour of different polypeptide chains *in vivo* (Gething & Sambrook, 1992). Thus, Leistler and Perham do not teach or suggest how to increase expression yields of soluble protein domains.

In the case of antibodies, two chains comprising several domains dimerize, each domain consisting of a  $\beta$ -barrel whose two  $\beta$ -sheets are held together by a disulphide bond, forming the so-called immunoglobulin fold. Two domains, one variable domain (VL) and one constant domain (CL) are adjacent along the longitudinal axis in the light chain (VL-CL), and four domains, one variable domain (VH) and three constant domain (CH1 to CH3) are adjacent along the longitudinal axis in the heavy chain (VH-CH1-CH2-CH3). In the dimer formed by chains a and b, two such domains associate laterally: VL<sub>a</sub> with VH<sub>a</sub>, CL<sub>a</sub> with CH1<sub>a</sub>, VL<sub>b</sub> with VH<sub>b</sub>, CL<sub>b</sub> with CH1<sub>b</sub>, CH2<sub>a</sub> with CH2<sub>b</sub> and CH3<sub>a</sub> with CH3<sub>b</sub>. In WO 92/01787 (Johnson et al., 1992), it is taught that isolated single domains, e.g. VH, can be modified in the former VL/VH interface region by exchanging hydrophobic residues by hydrophilic ones without changing the specificity of the parent domain. The rationale for WO 92/01787 was the

-3-

assumption that exposed hydrophobic residues might lead to non-specific binding, interaction with surfaces and decreased stability. Data for increase in binding specificity was given, but increase in expression level was not shown. Furthermore, WO 92/01787 would not be applicable to any antibody fragment containing the complete antigen binding site, as it must contain VL and VH.

In the case of T cell receptors, two chains ( $\alpha$  and  $\beta$ ) dimerize, each consisting of a variable (V) and a constant (C) domain with the immunoglobulin fold, and one transmembrane domain. In each chain, the variable and constant domains are adjacent along the longitudinal axis in the chains ( $V\alpha-C\alpha$ ;  $V\beta-C\beta$ ) and associate laterally with the corresponding domains of the second chain ( $V\alpha-V\beta$ ;  $C\alpha-C\beta$ ).

Various other molecules of the immunoglobulin superfamily, such as CD2, CD4, CD16, CD22, comprise only one chain, wherein two or more domains (variable and/or constant) with the immunoglobulin fold are adjacent along the longitudinal axis in the chains.

The present inventors have found that expression problems are largely associated with a part of the molecule that has hitherto not been regarded relevant for expression studies and which comprises the interface between adjacent domains within an immunoglobulin chain. This surprising finding forms the basis of the present invention, which provides a general solution to the problems associated with production of domains or fragments of the immunoglobulin superfamily (IgSF), especially antibody fragments, which exhibit poor solubility or reduced levels of expression.

### **Detailed Description of the Invention**

In addition to lateral interactions between domains of different chains described above, there are well documented contacts between adjacent domains within individual chains along the longitudinal axis. For example, in the case of an antibody (Lesk & Chothia, 1988), the "bottom" of VL makes contact with the "top" of CL, and, in a similar manner there are contacts between VH and CH1. The contacts at these inter-domain interfaces are probably essential for the compact arrangement of the Fab fragment, and, as is typical for such contacts, are at least partially hydrophobic in nature (Lesk & Chothia, 1988).

The basis of the present invention is the surprising finding that the solubility (and hence the yield) of antibody fragments comprising at least one domain can be dramatically increased by decreasing the hydrophobicity of former interfaces at the "end" of said domain, where it would normally adjoin a second domain within a chain in a larger antibody fragment or full antibody. This is surprising and could not have been predicted from the prior art (WO 92/01787), because the size of the longitudinal interface, for example, in a scFv fragment, is much smaller than that between VH and VL, and therefore, the amino acids which make up the interfaces between VH and CH1 or between VL and CL in a Fab fragment represent a much smaller proportion of the total surface area of the scFv molecule, and would accordingly be expected to play less of a role in determining the physical properties of the molecule.

The present invention has the additional advantage that because the alterations effected in the molecules that lead to said decreased hydrophobicity of former interfaces are located at the most distant part of the domain from the CDRs, applying the invention is unlikely to have a deleterious effect on the binding properties of the molecule. This is not the case in WO 92/01787, where at least one modification is close to the CDRs and may therefore be expected to have an impact on antigen binding. Furthermore, WO 92/01787 cannot be applied to VL/VH heterodimers, as explained above.

The present invention relates to a modified immunoglobulin superfamily (IgSF) domain or fragment which differs from a parent IgSF domain or fragment in that the region which comprised or would comprise the interface with a second domain adjoined to said parent IgSF domain or fragment within the protein chain of a larger IgSF fragment or a full IgSF protein, and which is exposed in said parent IgSF domain or fragment in the absence of said second domain, is made more hydrophilic by modification.

In the context of the present invention, the term immunoglobulin superfamily (IgSF) domain refers to those parts of members of the immunoglobulin superfamily which are characterized by the immunoglobulin fold, said superfamily comprising the immunoglobulins or antibodies, and various other proteins such as T-cell receptors or integrins. The term IgSF fragment refers to any portion of a member of the immunoglobulin superfamily, said portion comprising at least one IgSF domain. The term adjoining domain refers to a domain which is contiguous with a first domain. The term interface refers to a region of said first domain where interaction with the adjoining domain takes place. The terms hydrophobic and hydrophilic refer to a

physical property of amino acids, which can be estimated quantitatively: tabulated values of hydrophobicity for the twenty naturally-occurring amino acids are available (Nozaki & Tanford, 1971; Casari & Sippl, 1992; Rose & Wolfenden, 1993).

The residues to be modified can be identified in a number of ways. For example, in one way, the solvent accessibilities (Lee & Richards, 1971) of hydrophobic interface residues in said parent IgSF fragment compared to said larger IgSF fragment or full IgSF protein are calculated, with high accessibilities indicating highly exposed residues. In a second way, the number of van der Waals contacts of hydrophobic interface residues in said larger IgSF fragment or full IgSF protein is calculated. A large number for a residue of said parent domain indicates that it will be highly solvent-exposed in the absence of an adjoining domain. There are other ways of calculating or determining residues to be modified according to the present invention, and one of ordinary skill in the art will be able to identify and practice these ways.

By analyzing computer models of said parent IgSF fragment, interactions of said highly exposed residues within the fragment can be identified. Such interactions could stabilize the parent IgSF fragment. Residues, which interact closely with other hydrophobic residues and which can be identified by anyone of ordinary skill in the art, should not preferentially be mutated.

The modification referred to above may be effected in a number of ways which are well known to one skilled in the art. In a preferred embodiment, the modification is a substitution of one or more amino acids at the exposed interface, identified as described above, with amino acids which are more hydrophilic. Alternatively, one or more amino acids can be inserted in said interface, or one or more amino acids can be deleted from said interface, so as to increase its overall hydrophilicity. Furthermore, any combination of substitution, insertion and deletion can be effected to reduce the hydrophobicity of said interface. Also comprised by the present invention is the possibility that the substitution or insertion comprises amino acids with a relatively high hydrophobicity value, or that the deletion comprises amino acids with relatively low hydrophobicity value, as long as the overall hydrophilicity value is increased in said interface region. Modifications such as substitution, insertion and deletion can be effected using standard methods which are well known to practitioners skilled in the art. By way of example, the skilled artisan can use either site-directed or PCR-based mutagenesis (Ho et al., 1989; Kunkel et al., 1991; Trower, 1994; Viville, 1994), or total gene synthesis (Prodromou &

Pearl, 1992) to effect the necessary modification(s). In a further embodiment, the mutations may be obtained by random mutagenesis and screening of random mutants, using a suitable expression and screening system (see, for example, Stemmer, 1994; Cramer et al., 1996).

In a preferred embodiment, the amino acid(s) which replace(s) the more hydrophobic amino acids include Asn, Asp, Arg, Gln, Glu, Gly, His, Lys, Ser, and Thr. These are among the more hydrophilic of the 20 naturally-occurring amino acids, and have proven to be particularly effective in the application of the present invention. Said amino acids, alone or in combination, or in combination with other amino acids, can also be used to form the above mentioned insertion which makes the interface region more hydrophilic.

The parent IgSF domain or fragment referred to above can be one of several different types. In a preferred embodiment, said parent domain or fragment is derived from an antibody. In one embodiment, said parent antibody fragment comprises an Fv fragment. In this context, the term Fv fragment refers to a complex comprising the VL (variable light) and VH (variable heavy) portions of the antibody molecule. In a further embodiment, the parent antibody fragment may be a single-chain Fv fragment (scFv; Bird et al., 1988; Huston et al., 1988), in which the VL and VH chains are joined, in either a VL-VH, or VH-VL orientation, by a peptide linker. In yet a further embodiment, the parent antibody fragment may be an Fv fragment stabilized by an inter-domain disulphide bond. This is a structure which can be made by engineering into each chain a single cysteine residue, wherein said cysteine residues from two chains become linked through oxidation to form a disulphide (Glockshuber et al., 1990; Brinkmann et al., 1993).

In a most preferred embodiment, the interface region of the variable domains mentioned above comprises residues 9, 10, 12, 15, 39, 40, 41, 80, 81, 83, 103, 105, 106, 106A, 107, 108 for VL, and residues 9, 10, 11, 13, 14, 41, 42, 43, 84, 87, 89, 105, 108, 110, 112, 113 for VH according to the Kabat numbering system (Kabat et al., 1991). Said numbering system was established for the sequences of whole antibodies, but can be adapted correspondingly to describe the sequences of isolated antibody domains or antibody fragments, even in the case of scFv fragments, where VL and VH are connected via a peptide linker, and where the protein sequence from N- to C-terminus has to be numbered differently. This means that the Kabat numbering system is used in the present invention as a sequence description relative to the

-7-

existing data on antibody sequences, not as an absolute description of actual positions within the antibody fragment sequences of interest.

In a further embodiment, said parent antibody fragment comprises a Fab fragment. In this context, the term Fab refers to a complex comprising the VL-CL (variable and constant light) and VH-CH1 (variable and first constant heavy) portions of the antibody molecule, and the term interface region refers to a region in the first constant domain of the heavy chain (CH1) which is, or would be adjoined to, the CH2 domain in a larger antibody fragment or full antibody.

In a still further embodiment, said parent IgSF fragment is a fusion protein of any of said domains or fragments and another protein domain, derived from an antibody or any other protein or peptide. The advent of bacterial expression of antibody fragments has opened the way to the construction of proteins comprising fusions between antibody fragments and other molecules. A further embodiment of the present invention relates to such fusion proteins by providing for a DNA sequence which encodes both the modified IgSF domain or fragment, as described above, as well as an additional moiety. Particularly preferred are moieties which have a useful therapeutic function. For example, the additional moiety may be a toxin molecule which is able to kill cells (Vitetta et al., 1993). There are numerous examples of such toxins, well known those skilled in the art, such as the bacterial toxins *Pseudomonas* exotoxin A, and diphtheria toxin, as well as the plant toxins ricin, abrin, modeccin, saporin, and gelonin. By fusing such a toxin to an antibody fragment, the toxin can be targeted to, for example, diseased cells, and thereby have a beneficial therapeutic effect. Alternatively, the additional moiety may be a cytokine, such as IL-2 (Rosenberg & Lotze, 1986), which has a particular effect (in this case a T-cell proliferative effect) on a family of cells. In a further preferred embodiment, the additional moiety is at least part of a surface protein which may direct the fusion protein to the surface of an organism, for example, a cell or a phage, and thereby displays the IgSF partner. Preferably, the additional moiety is at least part of a coat protein of filamentous bacteriophages, most preferably of the geneIII protein. In a further embodiment, the additional moiety may confer on its IgSF partner a means of detection and/or purification. For example, the fusion protein could comprise the modified IgSF domain or fragment and an enzyme commonly used for detection purposes, such as alkaline phosphatase (Blake et al., 1984). There are numerous other moieties which can be used as detection or

purification tags, which are well known to the practitioner skilled in the art. Particularly preferred are peptides comprising at least five histidine residues (Hochuli et al., 1988), which are able to bind to metal ions, and can therefore be used for the purification of the protein to which they are fused (Lindner et al., 1992). Also provided for by the invention are additional moieties such as the commonly used c-myc and FLAG tags (Hopp et al., 1988; Knappik & Plückthun, 1994).

By engineering one or more fused additional domains, IgSF domains or fragments can be assembled into larger molecules which also fall under the scope of the present invention. To the extent that the physical properties of the IgSF domain or fragment determine the characteristics of the assembly, the present invention provides a means of increasing the solubility of such larger molecules. For example, mini-antibodies (Pack, 1994) are dimers comprising two antibody fragments, each fused to a self-associating dimerization domain. Dimerization domains which are particularly preferred include those derived from a leucine zipper (Pack & Plückthun, 1992) or helix-turn-helix motif (Pack et al., 1993).

All of the above embodiments of the present invention can be effected using standard techniques of molecular biology known to anyone skilled in the art.

The compositions described above may have utility in any one of a number of settings. Particularly preferred are diagnostic and therapeutic compositions.

The present invention also provides methods for making the compositions and compounds comprised therein described above. Particularly preferred is a method comprising the following steps:

- i) analyzing the interface region of an IgSF domain for hydrophobic residues which are solvent-exposed using either a solvent-accessibility approach (Lee & Richards, 1971), analysis of van der Waals interactions in the interface region, or similar methods which are well known to one skilled in the art.
- ii) identifying one or more of the hydrophobic residues to be substituted by more hydrophilic residues, or one or more positions where hydrophilic residues or amino acid stretches enhancing the overall hydrophilicity of the interface region can be inserted into said interface region, or one or more positions where hydrophobic residues or amino acid

stretches enhancing the overall hydrophobicity of the interface region can be deleted from said interface region, or any combination of said substitutions, said insertions, and said deletions to give one or more mutants of said parent IgSF domain.

- iii) preparing DNA encoding mutants of said IgSF domain, characterized by the changes identified in ii), by using e.g. conventional mutagenesis or gene synthesis methods, said DNA being prepared either separately or as a mixture.
- iv) introducing said DNA or DNA mixture in a vector system suitable for expression of said mutants.
- v) introducing said vector system into suitable host cells and expressing said mutant or mixture of mutants.
- vi) identifying and characterizing mutants which are obtained in higher yield in soluble form, and
- vii) if necessary, repeating steps iii) to vi) to increase the hydrophilicity of said identified mutant or mutants further.

The host referred to above may be any of a number commonly used in the production of heterologous proteins, including but not limited to bacteria, such as *E. coli* (Ge et al. 1995), or *Bacillus subtilis* (Wu et al., 1993), fungi, such as yeasts (Horwitz et al., 1988; Ridder et al., 1995) or filamentous fungus (Nyyssönen et al., 1993), plant cells (Hiatt, 1990; Hiatt & Ma, 1993; Whitelam et al., 1994), insect cells (Potter et al., 1993; Ward et al., 1995), or mammalian cells (Trill et al., 1995).

The invention also relates to a method for the production of an IgSF domain or fragment of the invention comprising culturing a host cell of the invention and isolating said domain or fragment.

The invention is now demonstrated by the following examples, which are presented for illustration only and are not intended to limit the scope of the invention.



## Examples

### i) Abbreviations

Abbreviations: CDR: complementarity determining region; dsFv: disulfide-linked Fv fragment; IMAC: immobilized metal ion affinity chromatography; IPTG: isopropyl- $\beta$ -D-thiogalactopyranoside; i/s: ratio insoluble/soluble; H(X): heavy chain residue number X; L(X): light chain residue number X; NTA: nitrilo-triacetic acid; OD<sub>550</sub>: optical density at 550 nm; PDB: protein database; scFv: single-chain Fv fragment; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; v/c: variable/constant; wt: wild type

### ii) Material and Methods

#### (a) Calculation of solvent accessibility

Solvent accessible surface areas for 30 non-redundant Fab fragments and the Fv fragments derived from these by deleting the constant domain coordinates from the PDB file were calculated using the latest version, as of March 1996, of the program NACCESS (<http://www.biochem.ucl.ac.uk/~roman/naccess/naccess>) based on the algorithm described by Lee & Richards (1971).

#### (b) scFv gene synthesis

The single-chain Fv fragment (scFv) in the orientation V<sub>L</sub>-linker-V<sub>H</sub> of the antibody 4-4-20 (Bedzyk et al., 1990) was obtained by gene synthesis (Prodromou and Pearl, 1992). The V<sub>L</sub> domain carries a three-amino acid long FLAG tag (Knappik and Plückthun, 1994). We have used two different linkers with a length of 15 (Gly<sub>4</sub>Ser)<sub>3</sub> and 30 amino acids (Gly<sub>4</sub>Ser)<sub>6</sub>, respectively. The gene so obtained was cloned into a derivative of the vector pIG6 (Ge et al., 1995). The mutant antibody fragments were constructed by site-directed mutagenesis (Kunkel et al., 1987) using single-stranded DNA and up to three oligonucleotides per reaction.

### (c) Expression

Growth curves were obtained as follows: 20 ml of 2xYT medium containing 100 µg/ml ampicillin and 25 µg/ml streptomycin were inoculated with 250 µl of an overnight culture of *E. coli* JM83 harboring the plasmid encoding the respective antibody fragment and incubated at 24.5°C until an OD<sub>550</sub> of 0.5 was reached. IPTG (Biomol Feinchemikalien GmbH) was added to a final concentration of 1 mM and incubation was continued for 3 hours. The OD was measured every hour, as was the β-lactamase activity in the culture supernatant to quantify the degree of cell leakiness. Three hours after induction an aliquot of the culture was removed and the cells were lysed exactly as described by Knappik and Plückthun (1995). The β-lactamase activity was measured in the supernatant, in the insoluble and in the soluble fraction. The fractions were assayed for antibody fragments by reducing SDS-PAGE, with the samples normalized to OD and β-lactamase activity to account for possible plasmid loss as well as for cell leakiness. The gels were blotted and immunostained using the FLAG antibody M1 (Prickett et al., 1989) as the first antibody, an Fc-specific anti-mouse antiserum conjugated to horseradish peroxidase (Pierce) as the second antibody, using a chemoluminescent detection assay described elsewhere (Ge et al., 1995).

### (d) Purification

Mutant scFv fragments were purified by a two-column procedure. After French press lysis of the cells, the raw *E. coli* extract was first purified by IMAC (Ni-NTA superflow, Qiagen) (20 mM HEPES, 500 mM NaCl, pH 6.9; step gradient of imidazole 10, 50 and 200 mM) (Lindner et al., 1992) and, after dialyzing the IMAC eluate against 20 mM MES pH 6.0, finally purified by cation exchange chromatography (S-Sepharose fast flow column, Pharmacia) (20 mM MES, pH 6.0; salt gradient 0-500 mM NaCl). Purity was controlled by Coomassie stained SDS-PAGE. The functionality of the scFv was tested by competition ELISA.

Because of its very poor solubility in the periplasmic system, the wt 4-4-20 was expressed as cytoplasmic inclusion bodies in the T7-based system (Studier & Moffatt, 1986; Ge et al., 1995). The refolding procedure was carried out as described elsewhere (Ge et al., 1995). For

-12-

purification, the refolding solution (2 l) was loaded over 10 h without prior dialysis onto a fluorescein affinity column, followed by a washing step with 20 mM HEPES, 150 mM NaCl, pH 7.5. Two column volumes of 1 mM fluorescein (sodium salt, Sigma Chemicals Co.) pH 7.5 were used to elute all functional scFv fragment. Extensive dialysis (7 days with 12 buffer changes) was necessary to remove all fluorescein. All purified scFv fragments were tested in gel filtration (Superose-12 column, Pharmacia SMART-System, 20 mM HEPES, 150 mM NaCl, pH 7.5).

(e)  $K_D$  determination by fluorescence titration

The concentrations of the proteins were determined photometrically using an extinction coefficient calculated according to Gill and von Hippel (1989). Fluorescence titration experiments were carried out by taking advantage of the intensive fluorescence of fluorescein. Two ml of 20 mM HEPES, 150 mM NaCl, pH 7.5 containing 10 or 20 nM fluorescein were placed in a cuvette with integrated stirrer. The excitation wavelength was 485 nm, emission spectra were recorded from 490 to 530 nm. Purified scFv (in 20 mM HEPES, 150 mM NaCl, pH 7.5) was added in 5 to 100  $\mu$ l aliquots, and after a 3 min equilibration time a spectrum was recorded. All spectra were recorded at 20°C. The maximum of emission at 510 nm was used for determining the degree of complexation of scFv to fluorescein, seen as quenching as a function of the concentration of the antibody fragment. The  $K_D$  value was determined by Scatchard analysis.

(f) Equilibrium denaturation measurement

Equilibrium denaturation curves were obtained by denaturation of 0.2  $\mu$ M protein in HEPES buffered saline (HBS) buffer (20 mM HEPES, 150 mM NaCl, 1 mM EDTA, pH 7.5) and increasing amounts of urea (1.0-7.5 M; 20 mM HEPES, 150 mM NaCl, pH 7.4; 0.25 M steps) in a total volume of 1.7 ml. After incubating the samples at 10°C for 12 hours and an additional 3 hours at 20°C prior to measurements, the fluorescence spectra were recorded at 20°C from 320-360 nm with an excitation wavelength of 280 nm. The emission wavelength of the fluorescence peak shifted from 341 to 347 nm during denaturation and was used for determining the fraction of unfolded molecules. Curves were fitted according to Pace (1990).

(g) Thermal denaturation

For measuring the thermal denaturation rates, purified scFv was dissolved in 2 ml HBS buffer to a final concentration of 0.5  $\mu$ M. The aggregation was followed for 2.5 h at 40°C and at 44°C by light scattering at 400 nm.

iii) Results

(a) Comparison of known antibody sequences

Compared to other domain/domain interfaces in proteins, the interface between immunoglobulin variable and constant domains is not very tightly packed. A comparison of 30 non-redundant Fab structures in the PDB database showed that between the light chain variable and constant domain an area of  $410 \pm 90 \text{ \AA}^2$  per domain is buried, while the heavy chain variable and constant domains interact over an area of  $710 \pm 180 \text{ \AA}^2$ . Some, but not all of the interface residues are hydrophobic, predominantly aliphatic. Generally, sequence conservation of the residues contributing to the v/c domain interface is not particularly high. Still, the v/c domain interface shows up as a marked hydrophobic patch on the surface of an Fv fragment (Fig. 1).

Solvent accessible surface areas for 30 non-redundant Fab fragments and their corresponding Fv fragments (derived from the Fab fragment by deleting the constant domain coordinates from the PDB file) were calculated using the program NACCESS (Lee & Richards, 1971). Residues participating in the v/c domain interface were identified by comparing the solvent-accessible surface area of each amino acid side chain in the context of an Fv fragment to its accessible surface in the context of a Fab fragment. Figure 2 shows a plot of the relative change in side chain accessibility upon deletion of the constant domains as a function of sequence position. Residues which show a significant reduction of side chain accessibility are also highlighted in the sequence alignment. To assess sequence variability in the positions identified in Figure 2, the variable domain sequences collected in the Kabat database (status March 1996) were analyzed (Table 1). Of the 15 interface residues identified in the VL

-14-

domain of the antibody 4-4-20 (Fig. 1 and Table 1). L9(leu), L12(pro), L15(leu), L40(pro), L83(leu), and L106(ile) are hydrophobic and therefore candidates for replacement. Of the 16 interface residues in the V<sub>H</sub> domain, H111(leu), H114(pro), H141(pro), H84(val), H87(met) and H89(ile) were identified as possible candidates for substitution by hydrophilic residues in the scFv fragment of the antibody 4-4-20 (Fig. 1 and Table 1).

Not all of these hydrophobic residues are equally good candidates for replacements, however. While residues which are hydrophobic in one particular sequence but hydrophilic in many other sequences may appear most attractive, the conserved hydrophobic residues listed in Table 1 have also been investigated, since the evolutionary pressure which kept these conserved residues acted on the Fab fragment within the whole antibody, but not the isolated Fv portion. In this study, we did not replace the proline residues since pro L40 and pro H41 form the hairpin turns at the bottom of the framework II region, while the conserved V<sub>L</sub> cis-proline L8 and proline residues H19 and H14 determine the shape of framework I of the immunoglobulin variable domains.

Excluding prolines, this leaves residues L9 (leu in 4-4-20, ser in most kappa chains), L15 (leu, usually hydrophobic), L83 (leu, usually val or phe) and L106 (Ile, as in 86% of all kappa chains) in the V<sub>L</sub> domain and H111 (leu as in 60% of all heavy chains), H84 (val, in other V<sub>H</sub> domains frequently ala or ser), H87 (met, usually ser) and H89 (ile, most frequently val) in V<sub>H</sub> as possible candidates for replacement in the 4-4-20 scFv fragment.

#### (b) Mutations in the 4-4-20 scFv

For the 4-4-20 scFv fragment some of the crucial residues identified in the sequence analysis described above are already hydrophilic, but nevertheless 9 residues are of hydrophobic nature (including pro12 in the light chain) (Table 1). We chose three residues for closer analysis by mutations.

Leu15 in V<sub>L</sub> is a hydrophobic amino acid in 98 % of all kappa chains (Table 1). Leu11 is conserved in V<sub>H</sub> (Table 1) and is involved in v/c interdomain contacts (Lesk & Chothia, 1988). In contrast, valine occurs very infrequently at position H84; mainly found at this

position are threonine or serine and alanine (Table 1). As can be seen in Figure 1, val84 is contributing to a large hydrophobic patch at the newly exposed surface of V<sub>H</sub>. All three positions were mutated into acidic residues, and L11 was also changed to asparagine (Table 2).

The scFv fragment was tested and expressed with two different linkers, the 15-mer linker (Gly4Ser)<sub>3</sub> (Huston et al., 1995) and the same motif extended to 30 amino acids (Gly4Ser)<sub>6</sub>. All mutations were tested in both constructs. The *in vivo* results of the different mutations on solubility were identical, and therefore only the results of the 30-mer linker are described in more detail. The periplasmic expression experiments were carried out at 24.5°C, and all constructs were tested for soluble and insoluble protein by immunoblotting. The ratio of insoluble to soluble (i/s) protein was determined for every mutant. In Figure 3 A-D, insoluble (lane 1) and soluble (lane 2) fractions of the wt scFv are shown. Nearly no soluble material occurs in periplasmic expression, which is consistent with previous reports of Bedzyk et al. (1990) and Denzin et al. (1991), who described earlier that the periplasmic expression of the wt scFv leads mainly to periplasmic inclusion bodies.

The single point mutation L15E in V<sub>L</sub> (Flu1) shows no effect on the ratio i/s when compared with the wt (Fig. 3A, lane 3, 4). Mutating leu at position 11 in the heavy chain to asparagine (Flu2) also shows nearly no effect compared to the wt, whereas the substitution with aspartic acid (Flu3) changes the i/s ratio to more soluble protein, but still this effect is not very dramatic. In contrast, the point mutation at position 84 (Flu4, Fig. 3B, lane 3, 4 and Fig 3D, lane 3, 4) had a dramatic influence on the solubility of the scFv fragment of the antibody 4-4-20. The ratio i/s is changed to about 1:1, resulting in a 25-fold increase of soluble protein compared to the wt.

The combination of V84D with L11N or L11D (Flu5, Flu6) also changes the ratio i/s compared to the wt, but this ratio compared to V84D alone is not improved further (Fig. 3B). Interestingly, the combination of Flu5 with the light chain mutation at position 15 (Flu9) leads to less soluble material (Fig. 3C lane 7,8) than Flu5 itself (Fig. 3B, lane 5, 6). The negative influence of the L15E mutations can also be seen in Flu8 (Fig. 3C, lane 5, 6) compared with

-16-

Flu3 (Fig. 3A, lane 7, 8). In Fig. 3D the comparison of the wt (lane 1, 2 and 5, 6) and Flu4 (lane 3, 4 and 7, 8) is shown in both the 15-mer and the 30-mer construct.

The negative effect of L15E can be rationalized by looking at a model of the 4-4-20 scFv fragment. L15 is forming a hydrophobic pocket together with residues A80, L83, and L106. Apparently, L15 stabilizes the scFv fragment by hydrophobic interactions with its closest neighbours. Thus the exchange L15E for making the scFv fragment more hydrophilic and more soluble is made at the expense of the fragment stability. The analysis of hydrophobic interactions within a fragment should thereby be used to choose the solvent-exposed residues to be mutated in the case of any other antibody fragment.

Combinations of various serine mutations in VH led to further improvements in the i/s ratio. The mutants FH15 (V84S, M87S, I89S) and FH20 (L11S, V84S, M87S, I89S) both showed more than 70% of soluble protein in immunoblots (data not shown).

The negative effect of L15E

#### (c) Functional expression and purification

The oligomerization of scFv fragments as a function of linker length has been investigated previously. A continuous decrease in the amount of dimer and multimer formation as a function of linker length has been reported (Desplancq et al., 1994; Whitlow et al., 1994). While the (Gly4Ser)<sub>3</sub> linker has been shown to lead to monomeric scFvs in many cases in the V<sub>H</sub>-V<sub>L</sub> direction, this is often not the case in the V<sub>L</sub>-V<sub>H</sub> direction. This is caused by an asymmetry in the V<sub>L</sub>/V<sub>H</sub> arrangement, leading to a longer distance between the end of V<sub>H</sub> and the N-terminus of V<sub>L</sub> than between C-terminus of V<sub>L</sub> and N-terminus of V<sub>H</sub> (Huston et al., 1995). Consequently, a linker of identical length may lead to different properties of the resulting molecules.

Since we have chosen to use the minimal perturbation FLAG (Knappik & Plückthun, 1994) at the N-terminus of V<sub>L</sub> in our constructs and thus the V<sub>L</sub>-linker-V<sub>H</sub> orientation, we have investigated the use of longer linkers. In the periplasmic expression in *E. coli* no difference between the 15-mer and the 30-mer linker in the corresponding mutants is visible (Fig. 3D), but when we attempted to purify the two Flu4 scFvs with long and short linker, a big

-17-

discrepancy between the two constructs was found. The purification of the Flu4 mutant (V84D) with the 15-mer linker leads to very small amounts of partially purified protein (about 0.015 mg per liter and OD; estimated from SDS-PAGE after IMAC purification), whereas the 30-mer linker construct gives about 0.3 mg per liter and OD of highly pure functional protein. All mutants with 30-mer linker were tested in gel filtration and found to be monomeric (data not shown).

For further *in vitro* characterization five mutants were purified with the 30-mer linker, V84D (Flu4), V84D/L11D (Flu6), L11D (Flu3), and the serine mutants FH15 and FH20 (see iii(b)). A two-step chromatography, first using IMAC and then cation-exchange chromatography, led to homogeneous protein. The i/s ratio of the antibody fragments (Fig. 3) was also reflected in the purification yield of functional protein. The highly soluble mutant Flu4 (V84D) (Fig. 3B lane 3, 4) yielded about 0.3 mg purified and functional protein per liter and OD, Flu6 (L11D/V84D) (Fig. 3B lane 7, 8) yielded about 0.25 mg per liter and OD and Flu3 (less soluble material on the blot in Fig. 3A lane 7, 8) yielded 0.05 mg per liter and OD. The serine mutants FH15 and FH20 yielded 0.3 mg and 0.4 mg per liter and OD, respectively. The wt scFv of the antibody 4-4-20 did not give any soluble protein at all in periplasmic expression with either linker, and it was therefore expressed as cytoplasmic inclusion bodies, followed by refolding *in vitro* and fluorescein affinity chromatography. The refolded wt scFv was shown by gel filtration to be monomeric with the 30-mer linker (data not shown).

#### (d) Biophysical properties of the mutant scFvs

Since we changed amino acids which are conserved, it cannot be excluded that changes at these positions may be transmitted through the structure and have an effect on the binding constant, even though they are very far from the binding site (Chatellier et al., 1996). To eliminate this possibility, we determined the binding constant of the mutants Flu3, Flu4, Flu6 and the wt scFv. Fluorescence titration was used to determine  $K_D$  in solution by using the quenching of the intrinsic fluorescence of fluorescein when it binds to the antibody. The fluorescence quenching at 510 nm was measured as a function of added scFv. The  $K_D$  values (Table 3 and Fig. 4) obtained for all three mutant scFvs and the wt scFv are very similar and



correspond very well to the recently corrected  $K_D$  of the monoclonal antibody 4-4-20 (Miklasz et al., 1995).

To determine whether the mutations had an influence on the thermodynamic stability of the protein we determined the equilibrium unfolding curves by urea denaturation. V84D mutant and the wt scFv were used for this analysis, and in Figure 5 an overlay plot is shown. The midpoint of both curves is at 4.1 M urea. Both curves were fitted by an algorithm for a two-state model described by Pace (1990), but the apparent small difference between the V84D mutant and the wt scFv is not of statistical significance.

Aggregation of folding intermediates could be another explanation for the different *in vivo* results between the mutant scFvs and the wt scFv (Fig.3). In the periplasm of *E. coli*, the protein concentrations are assumed to be rather high (van Wielink & Duine, 1990) and the aggregation effects could thus be pronounced. In order to estimate the aggregation behavior *in vitro*, we have measured the thermal aggregation rates at different temperatures. In Figure 6 it is clearly seen that the wt scFv is significantly aggregating already at 44°C, whereas the mutant V84D tends to aggregate more slowly. The wt scFv is thus clearly more aggregation prone than the mutant scFv. This is very similar to the observations made with different mutations on the antibody McPC603 (Knappik and Plückthun, 1995), where no correlation was found between equilibrium denaturation curves and expression behavior, but a good correlation was found with the thermal aggregation rates.

## Figures and Tables

Figure 1: Space-filling representation of the Fv fragment of the antibody 4-4-20

Figure 2: Variable/constant domain interface residues for V<sub>L</sub> (2a) and V<sub>H</sub> (2b). For 30 non-redundant Fab fragments taken from the Brookhaven Databank, the solvent accessible surface of the amino acid side chains was calculated in the context of

an Fv and of an Fab fragment. The plot shows the relative reduction in accessible surface upon contact with the constant domains (overlay plot for all 30 Fv fragments). In the sequence alignment, residues contributing to the v/c interface are highlighted. The symbols indicate the relative reduction of solvent accessible surface upon removing the constant domains (symbols: no symbol < 1%; ● < 20%; ■ < 40%; ▲ < 60%; ▼ < 80%, and ◆ ≥ 80%). Circles indicate those positions which are further analyzed (see Table 1).

Figure 3: Western blots showing the insoluble (i) and soluble (s) fractions of cell extracts, prepared as described in Material and Methods, expressing the scFv fragments of the antibody 4-4-20. The amino acids substituted in the various mutants are given in Table 2.

Figure 4: Scatchard plot of the fluorescence titration of fluorescein (20 nM) with antibody (4 to 800 nM), measured at 510 nm. The value  $r$  was obtained from  $(F-F_0)/(F_\infty-F_0)$ , where  $F$  is the measured fluorescein fluorescence at a given antibody concentration,  $F_0$  is the fluorescence in the absence of antibody and  $F_\infty$  when antibody is present in large excess. Note that  $r$  gives the saturation of fluorescein by antibody. (a) Titration of wt scFv. (b) titration of Flu4 (V84D).

Figure 5: An overlay plot of the urea denaturation curves is shown. (X) wt scFv. (o) Flu4.

Figure 6: Thermal denaturation time courses at 40 and 44°C for wt and Flu4 scFv fragment are shown. (a) wt scFv at 40°C. (b) Flu4 at 40°C. (c) Flu4 at 44°C. (d) wt scFv at 44°C.

Table 1: Sequence variability of residues contributing to the v/c interface: Residue statistics are based on the variable domain sequences in the Kabat database (March 1996). Sequences which were <90% complete were excluded from the analysis. Number of sequences analyzed: human VL kappa: 404 of 881, murine VL kappa: 1061 of 2239, human VL lambda: 223 of 409, murine VL lambda: 71 of 206, human VH: 663 of 1756, murine VH: 1294 of 3849. Position refers to the sequence position

-20-

according to Kabat et al. 1991. %exp. (Fab) to the relative side chain accessibility in an Fab fragment as calculated by the program NACCESS (NACCESS v2.0 by Simon Hubbard (<http://www.biochem.ucl.ac.uk/~roman/naccess/naccess.html>)). %exp. (ind.) to the relative side chain accessibility in the isolated VL or VH domain. %buried to the relative difference in side chain accessibility between Fv and Fab fragment. Consensus refers to the sequence consensus, and Distribution to the distribution of residue types.

Table 2: Mutations introduced in the scFv fragment of the antibody 4-4-20: Each line represents a different protein carrying the mutations indicated. The residues are numbered according to Kabat et al. (1991).

Table 3:  $K_D$  values of the different scFv mutants determined in fluorescence titration: The  $K_D$  values are given in nM, the error was calculated from the Scatchard analysis (Fig. 4). # determined by Miklasz et al. (1995)

## References

- Better, M., Chang, P., Robinson, R. & Horwitz, A.H. (1988). *E. coli* secretion of an active chimeric antibody fragment. *Science* **240**, 1041-1043.
- Bird, R.E., Hardman, K.D., Jacobson, J.W., Johnson, S., Kaufman, B.M., Lee S.M., Lee T., Pope S.H., Riordan G.S., & Whitlow M. (1988). Single-chain antigen-binding proteins [published erratum appears in (1989). *Science* **244**, 409]. *Science* **242**, 423-6.
- Bedzyk, W.D., Weidner, K.M., Denzin, L.K., Johnson, L.S., Hardman, K.D., Pantoliano, M.W., Asel, E.D. & Voss, E.W., Jr. (1990). Immunological and structural characterization of a high affinity anti-fluorescein single-chain antibody. *J. Biol. Chem.* **265**, 18615-18620.

- Blake, M.S., Johnston, K.H., Russel-Jones, G.J. & Gotschlich, E.C. (1984). A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Anal. Biochem.* **136**, 175-179.
- Brinkmann, U., Reiter, Y., Jung, S., Lee, B. & Pastan, I. (1993). A recombinant immunotoxin containing a disulfide-stabilized Fv fragment. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7538-7542.
- Carter, P., Kelley, R.F., Rodrigues, M.L., Snedecor, B., Covarrubias, M., Velligan, M.D., Wong, W.L.T., Rowland, A.M., Kotts, C.E., Carver, M.E., Yang, M., Bourell, J.H., Shepard, H.M. & Henner, D. (1992). High level *Escherichia coli* expression and production of a bivalent humanized antibody fragment. *Bio/Technology* **10**, 163-167.
- Casari, G. & Sippl, M.J. (1992). Structure-derived hydrophobic potential. Hydrophobic potential derived from X-ray structures of globular proteins is able to identify native folds. *J. Mol. Biol.*, **224**, 725-32.
- Chatellier, J., van Regenmortel, M.H.V., Vernet, T. & Altschuh, D. (1996). Functional mapping of conserved residues located at the V<sub>L</sub> and V<sub>H</sub> domain interface of an Fab. *J. Mol. Biol.*, *in press*.
- Denzin, L.K., Whitlow, M. & Voss, E.W., Jr. (1991). Single-chain site-specific mutation of fluorescein-amino acid contact residues in high affinity monoclonal antibody 4-4-20. *J. Biol. Chem.* **266**, 14095-14102.
- Desplancq, D., King, D.J., Lawson, A.D. & Mountain, A. (1994). Multimerization behaviour of single chain Fv variants for the tumour-binding antibody B72.3. *Protein Eng.* **7**, 1027-1033.
- Ge, L., Knappik, A., Pack, P., Freund, C. & Plückthun, A. (1995). *Expressing antibodies in Escherichia coli*. Antibody Engineering. A Practical Approach (Ed. C.A.K. Borrebaeck). IRL Press, Oxford, pp. 229-266.
- Gething, M.J. & Sambrook, J. (1992). Protein folding in the cell. *Nature* **355**, 33-45.
- Gill, S.C. & von Hippel, P.H. (1989). Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182**, 319-326.
- Glockshuber, R., Malia, M., Pfitzinger, I. & Plückthun, A. (1992). A comparison of strategies to stabilize immunoglobulin Fv-fragments. *Biochemistry* **29**, 1362-1366.
- Hiatt, A. (1990). Antibodies produced in plants. *Nature* **344**, 469-470.

- Hiatt, A. & Ma, J. K. (1993). Characterization and applications of antibodies produced in plants. *Int. Rev. Immunol.* **10**, 139-152.
- Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. & Pease, L.R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**, 51-9.
- Hochuli, E., Bannwarth, W., Döbeli, H., Gentz, R. & Stüber, D. (1988). Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. *Bio/Technology* **6**, 1321-1325.
- Hopp, T.P., Prickett, K.S., Price, V.L., Libby, R.T., March, C.J., Cerretti, D.P., Urdal, D.L. & Conlon, P.J. (1988). A short polypeptide marker sequence useful for recombinant protein identification and purification. *Bio/Technology* **6**, 1204-1210.
- Horwitz, A. H., Chang, C. P., Better, M., Hellstrom, K. E. & Robinson, R. R. (1988). Secretion of functional antibody and Fab fragment from yeast cells. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8678-8682.
- Huston, J., George, A.J.T., Tai, M., McCartney, J.E., Jin, D., Segal, D.M., Keck, P. & Oppermann, H. (1995). *Single-chain Fv design and production by preparative folding*. Antibody Engineering. A Practical Approach (Ed. C.A.K. Borrebaeck). IRL Press, Oxford, pp. 185-228.
- Huston, J.S., Levinson, D., Mudgett-Hunter, M., Tai, M.S., Novotny, J., Margolies, M.N., Ridge, R.J., Bruccoleri, R.E., Haber, E. & Crea, R. (1988). Protein engineering of antibody binding sites. recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* **85**, 5879-83.
- Johnson, K.S., Jackson, R.H. & Chiswell, D.J. (1992). Binding Domains. *PCT Application WO 92/01787*.
- Kabat, E.A., Wu, T.T., Perry, H.M., Gottesmann, K.S. & Foeller, C. (1991). Sequences of proteins of immunological interest. U.S. Dept. of Health and Human Services, Public Health Service, National Institutes of Health. NIH Publication 91-3242.
- Knappik, A. & Plückthun, A. (1994). An improved affinity tag based on the FLAG peptide for detection and purification of recombinant antibody fragments. *BioTechniques* **17**, 754-761.

- Knappik, A. & Plückthun, A. (1995). Engineered turns of a recombinant antibody improve its *in vivo* folding. *Protein Eng.* **8**, 81-89.
- Kunkel, T.A., Bebenek, K. & McClary, J. (1991). Efficient site-directed mutagenesis using uracil-containing DNA. *Methods in Enzymol.* **204**, 125-39.
- Kunkel, T.A., Roberts, J.D. & Zakour, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods in Enzymol.* **154**, 367-382.
- Lee, B. & Richards, F.M. (1971). The interpretation of protein structures: estimation of static accessibility. *J. Mol. Biol.* **55**, 379-400.
- Lesk, A.M. & Chothia, C. (1988). Elbow motion in the immunoglobulins involves a molecular ball-and-socket joint. *Nature (London)* **335**, 188-190.
- Leistler, B. & Perham, R.N. (1994). Solubilizing buried domain proteins: A self-assembling interface domain from glutathione reductase. *Biochemistry* **33**, 2773-2781.
- Lindner, P., Guth, B., Wülfing, C., Krebber, C., Steipe, B., Müller, F. & Plückthun, A. (1992). Purification of native proteins from the cytoplasm and periplasm of *Escherichia coli* using IMAC and histidine tails: a comparison of proteins and protocols. *Methods: A Companion to Methods Enzymol.* **4**, 41-56.
- Miklasz, S.D., Gulliver, G.A. & Voss, E.W., Jr. (1995). High-affinity rat anti-fluorescein monoclonal antibody with unique fine specificity properties including differential recognition of dynamic ligand analogues. *J. Mol. Recognition* **8**, 258-269.
- Munro, S. & Pelham, H.R.B. (1986). An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell*, **46**, 291-300.
- Nozaki, Y. & Tanford, C. (1971). The solubility of amino acids and two glycine peptides in aqueous ethanol and dioxane solutions. Establishment of a hydrophobicity scale. *J. Biol. Chem.* **246**, 2211-7.
- Nyyssönen, E., Penttilä, M., Harkki, A., Saloheimo, A., Knowles, J. K. & Keranen, S. (1993). Efficient production of antibody fragments by the filamentous fungus *Trichoderma reesei*. *Bio/Technology* **11**, 591-595.
- Pace, C.N. (1990). Measuring and increasing protein stability. *Trends Biotechnol.* **8**, 93-98.
- Pack, P. & Plückthun, A. (1992). Miniantibodies: use of amphipathic helices to produce functional, flexibly linked dimeric Fv fragments with high avidity in *Escherichia coli*. *Biochemistry* **31**, 1579-1584.

- Pack, P., Kujau, M., Schroeckh, V., Knüpfer, U., Wenderoth, R., Riesenberger, D. & Plückthun, A. (1993). Improved bivalent miniantibodies, with identical avidity as whole antibodies, produced by high cell density fermentation of in *Escherichia coli*. *Bio/Technology* **11**, 1271-1277.
- Pack, P. (1994). Mini-Antikörper: Bivalente, tetravalente und bispezifische Immunglobuline aus *E. coli*. Ph.D. thesis, Ludwig-Maximilians-Universität München.
- Plückthun, A. (1992). Mono- and bivalent antibody fragments produced in *Escherichia coli*: engineering, folding and antigen binding. *Immun. Rev.* **130**, 151-188.
- Plückthun, A., Krebber, A., Krebber, C., Horn, U., Knüpfer, U., Wenderoth, R., Nieba, L., Proba, K. & Riesenberger, D. (1996). *Producing antibodies in Escherichia coli: From PCR to fermentation*. A practical approach. Antibody Engineering (Ed. J. McCafferty). IRL Press, Oxford, pp. 203-252.
- Potter, K. N., Li, Y. & Capra, J. D. (1993). Antibody production in the baculovirus expression system. *Int. Rev. Immunol.* **10**, 103-112.
- Prickett, K.S., Amberg, D.C. & Hopp, T.P. (1989). A calcium-dependent antibody for identification and purification of recombinant proteins. *BioTechniques* **7**, 580-589.
- Prodromou, C. & Pearl, L.H. (1992). Recursive PCR: a novel technique for total gene synthesis. *Protein Eng.* **5**, 827-829.
- Ridder, R., Schmitz, R., Legay, F. & Gram, H. (1995). Generation of rabbit monoclonal antibody fragments from a combinatorial phage display library and their production in the yeast *Pichia pastoris*. *Bio/Technology* **13**, 255-260.
- Rose, G.D. & Wolfenden, R. (1993). Hydrogen bonding, hydrophobicity, packing, and protein folding. *Annu Rev. Biophys. Biomol. Struct.* **22**, 381-415.
- Rosenberg, S.A. & Lotze, M.T. (1986). Cancer immunotherapy using interleukin-2 and interleukin-2 activated lymphocytes. *Ann. Rev. Immunol.* **4**, 681-709.
- Skerra, A. & Plückthun (1988). Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. *Science* **240**, 1038-1041.
- Studier, F.W. & Moffatt, B.A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**, 113-130.
- Trill, J. J., Shatzman, A. R. & Ganguly, S. (1995). Production of monoclonal antibodies in COS and CHO cells. *Curr. Opin. Biotechnol.* **6**, 553-560.

- Trower, M.K. (1994). Site-directed mutagenesis using a uracil-containing phagemid template. *Methods Mol. Biol.* **31**, 67-77.
- Ullrich, H.D., Patten, P.A., Yang, P.L., Romesberg, F.E. & Schultz, P.G. (1995). Expression studies of catalytic antibodies. *Proc. Natl. Acad. Sci. USA* **92**, 11907-11911.
- Van Wielink, J.E. & Duine, J.A. (1990). How big is the periplasmic space. *Trends Biochem. Sci.* **15**, 136-137.
- Vitetta, E.S., Thorpe, P.E. & Uhr, J. (1993). Immunotoxins: magic bullets or misguided missiles. *Immunol. Today* **14**, 253-259.
- Viville, S. (1994). Site-directed mutagenesis using a double-stranded DNA template. *Methods Mol. Biol.* **31**, 57-65.
- Ward, V. K., Kreissig, S. B., Hammock, B. D. & Choudary, P. V. (1995). Generation of an expression library in the baculovirus expression vector system. *J. Virol. Methods* **53**, 263-272.
- Whitelam, G. C., Cockburn, W. & Owen, M. R. (1994). Antibody production in transgenic plants. *Biochem. Soc. Trans.* **22**, 940-944.
- Whitlow, M., Filupa, D., Rollence, M.L., Feng, S. & Wood, J.F. (1994). Multivalent Fvs: characterization of single-chain Fv oligomers and preparation of a bispecific Fv. *Protein Eng.* **7**, 1017-1026.
- Wu, X. C., Ng, S. C., Near, R. I. & Wong, S. L. (1993). Efficient production of a functional single-chain antidigoxin antibody via an engineered *Bacillus subtilis* expression-secretion system. *Bio/Technology* **11**, 71-76.



**Claims**

1. A DNA sequence which encodes an immunoglobulin superfamily (IgSF) domain or fragment which differs from a parent IgSF domain or fragment in that the region which comprised or would comprise the interface with a second domain adjoined to said parent IgSF domain or fragment within the chain of a larger IgSF fragment or protein is made more hydrophilic by modification.
2. The DNA sequence according to claim 1 in which said modification is substitution of one or more amino acids at said interface with amino acids which are more hydrophilic.
3. The DNA sequence according to claim 1 in which said modification is insertion of one or more hydrophilic amino acids in said interface, or insertion of amino acids which increase the overall hydrophilicity in said interface, or deletion of one or more hydrophobic amino acids in said interface, or deletion of amino acids, said deletion leading to an increase in the overall hydrophilicity in said interface.
4. The DNA sequence according to claim 1 in which said modification consists of any two or more of:
  - a) substitution of one or more amino acids at said interface with amino acids which are more hydrophilic,
  - b) insertion of one or more hydrophilic amino acids in said interface, or insertion of amino acids which increase the overall hydrophilicity in said interface,
  - c) deletion of one or more hydrophobic amino acids in said interface, or deletion of amino acids, said deletion leading to an increase in the overall hydrophilicity in said interface.
5. The DNA sequence according to any of claims 2 to 4 in which said substituted or inserted amino acid is taken from the list Asn, Asp, Arg, Gln, Glu, Gly, His, Lys, Ser, and Thr.
6. The DNA sequence according to any of claims 1 to 5 in which said parent IgSF domain is part of an IgSF fragment.

-27-

7. The DNA sequence according to any of claims 1 to 6 in which said domain or fragment is derived from an antibody.
8. The DNA sequence according to claim 7 in which said fragment is a Fab fragment.
9. The DNA sequence according to claim 7 in which said fragment is an Fv fragment.
10. The DNA sequence according to claim 7 in which said fragment is a scFv fragment.
11. The DNA sequence according to claim 7 in which said fragment is an Fv stabilized by an inter-domain disulphide bond.
12. The DNA sequence according to any of claims 9 to 11 in which said interface region comprises residues 9, 10, 12, 15, 39, 40, 41, 80, 81, 83, 103, 105, 106, 106A, 107, 108 for VL, and residues 9, 10, 11, 13, 14, 41, 42, 43, 84, 87, 89, 105, 108, 110, 112, 113 for VH.
13. The DNA sequence according to any of claims 1 to 12, having a contiguous sequence which encodes one or more additional moieties.
14. The DNA sequence according to claim 13 in which at least one of said additional moieties is a toxin, a cytokine, or a reporter enzyme.
15. The DNA sequence according to claim 13 in which at least one of said additional moieties is at least part of a surface protein of an organism.
16. The DNA sequence according to claim 15 in which said organism is a filamentous bacteriophage.
17. The DNA sequence according to claim 16 in which said surface protein is the geneIII protein.
18. The DNA sequence according to claim 13 in which at least one of said additional moieties is capable of binding a metal ion.
19. The DNA sequence according to claim 18 in which at least one of said additional moieties comprises at least five histidines.
20. The DNA sequence according to claim 13 in which said moiety is a peptide.

-28-

21. The DNA sequence according to claim 20 in which said peptide is a labelling tag.
22. The DNA sequence according to claim 21 in which said labelling tag is c-myc or FLAG.
23. The DNA sequence according to claim 20 in which said peptide comprises an association domain which results in self-association of two or more of said antibody fragments.
24. The DNA sequence according to claim 23 in which said association domain is derived from a leucine zipper or from a helix-turn-helix motif.
25. The DNA sequence according to claim 20 in which said peptide comprises a first association domain which results in hetero-association of one or more of said antibody fragments with one or more peptides or proteins comprising a second hetero-association domain being able to associate with said first hetero-association domain.
26. A vector comprising a DNA sequence according to any of claims 1 to 25.
27. A host cell comprising a vector according to claim 26.
28. An IgSF domain or fragment, or a fusion protein comprising an IgSF domain or fragment, encoded by a DNA sequence according to any of claims 1 to 25, by a vector according to claim 26, or produced by a host cell according to claim 27.
29. A diagnostic composition comprising an IgSF domain or fragment, or a fusion protein comprising an IgSF domain or fragment, according to claim 28.
30. A therapeutic composition comprising an IgSF domain or fragment, or a fusion protein comprising an IgSF domain or fragment, according to claim 28.
31. A method for deriving a DNA sequence according to any of claims 1 to 25 which comprises the following steps:
  - i) analyzing the interface region of a parent IgSF domain for hydrophobic residues which are solvent-exposed.
  - ii) identifying one or more of the hydrophobic residues to be substituted by more hydrophilic residues, or one or more positions where hydrophilic residues or amino acid stretches enhancing the overall hydrophilicity of the interface region can be

-29-

inserted into said interface region, or one or more positions where hydrophobic residues or amino acid stretches enhancing the overall hydrophobicity of the interface region can be deleted from said interface region, or any combination of said substitutions, said insertions, and said deletions to give one or more mutants of said parent IgSF domain.

32. A method for making an IgSF domain or fragment, or a fusion protein comprising an IgSF domain or fragment, according to claim 28 which comprises the following steps:

i) deriving a DNA sequence according to claim 31,

ii) preparing DNA encoding said mutant or mutants, said DNA being prepared either separately or as a mixture,

iii) introducing said DNA or DNA mixture in a vector system suitable for expression of said mutant or mutants, said vector system optionally comprising one or more additional DNA sequences suitable for expression of additional IgSF domains or fragments, or one or more DNA sequences suitable for expression of a fusion protein comprising said mutant or mutants, or any combination of said additional DNA sequences,

iv) introducing said vector system into suitable host cells and expressing said mutant or mixture of mutants, or expressing said mutants or mixture of mutants in combination with the expression products of said additional DNA sequences,

v) identifying and characterizing one or more mutants, alone or in said combination, which are obtained in higher yield in soluble form, and

vi) if necessary, repeating steps ii) to vi) to increase the hydrophilicity of said identified mutant or mutants, alone or in said combination, further.

33. The method according to claim 32 in which said host is a bacterium, a fungus, a plant, an insect cell, or a cell-line of mammalian origin.

34. A method for the production of an IgSF domain or fragment of claim 28 comprising culturing a host cell of claim 27 and isolating said domain or fragment.

**Figure 1: Space filling representation of the Fv fragment of the antibody 4-4-20**

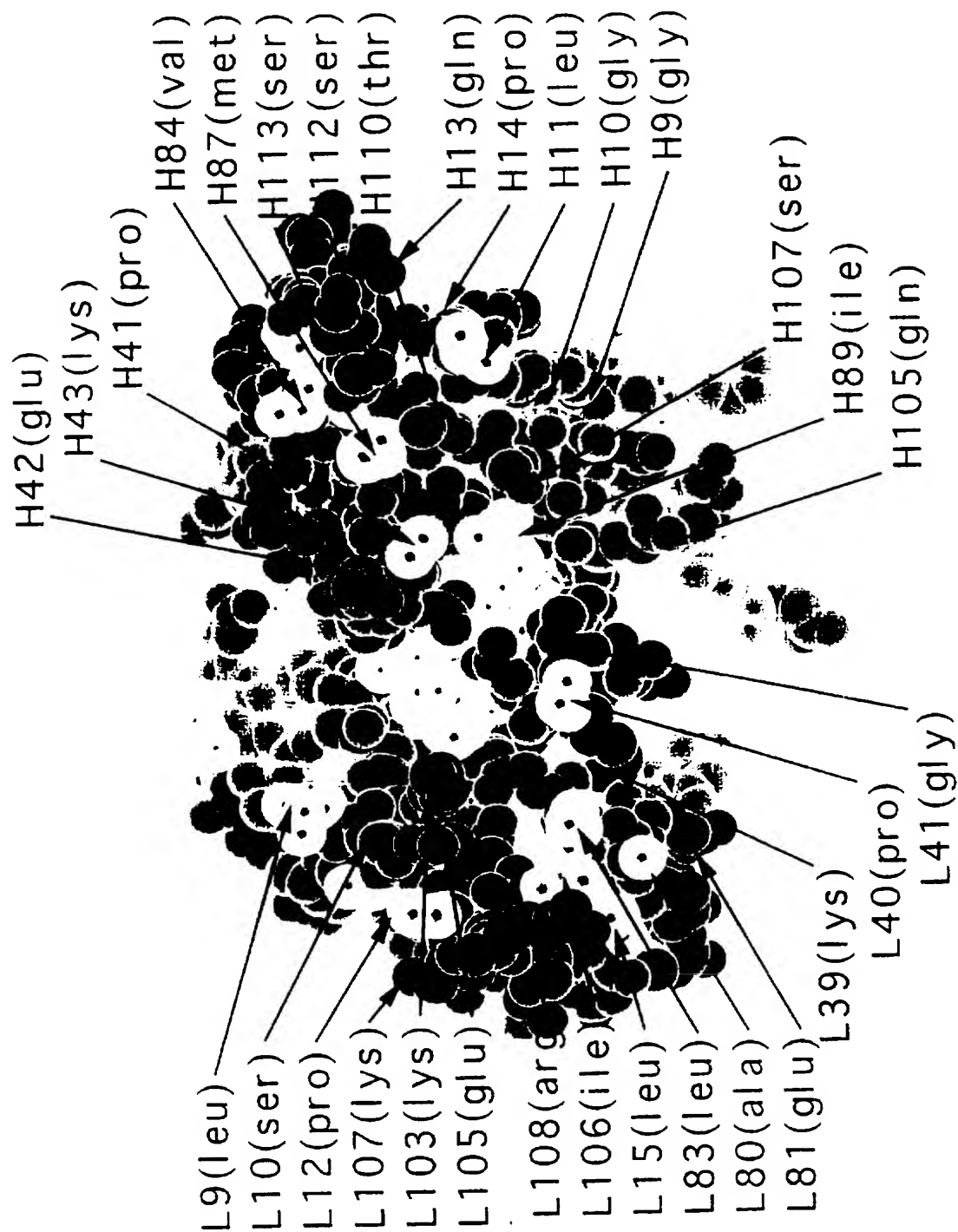
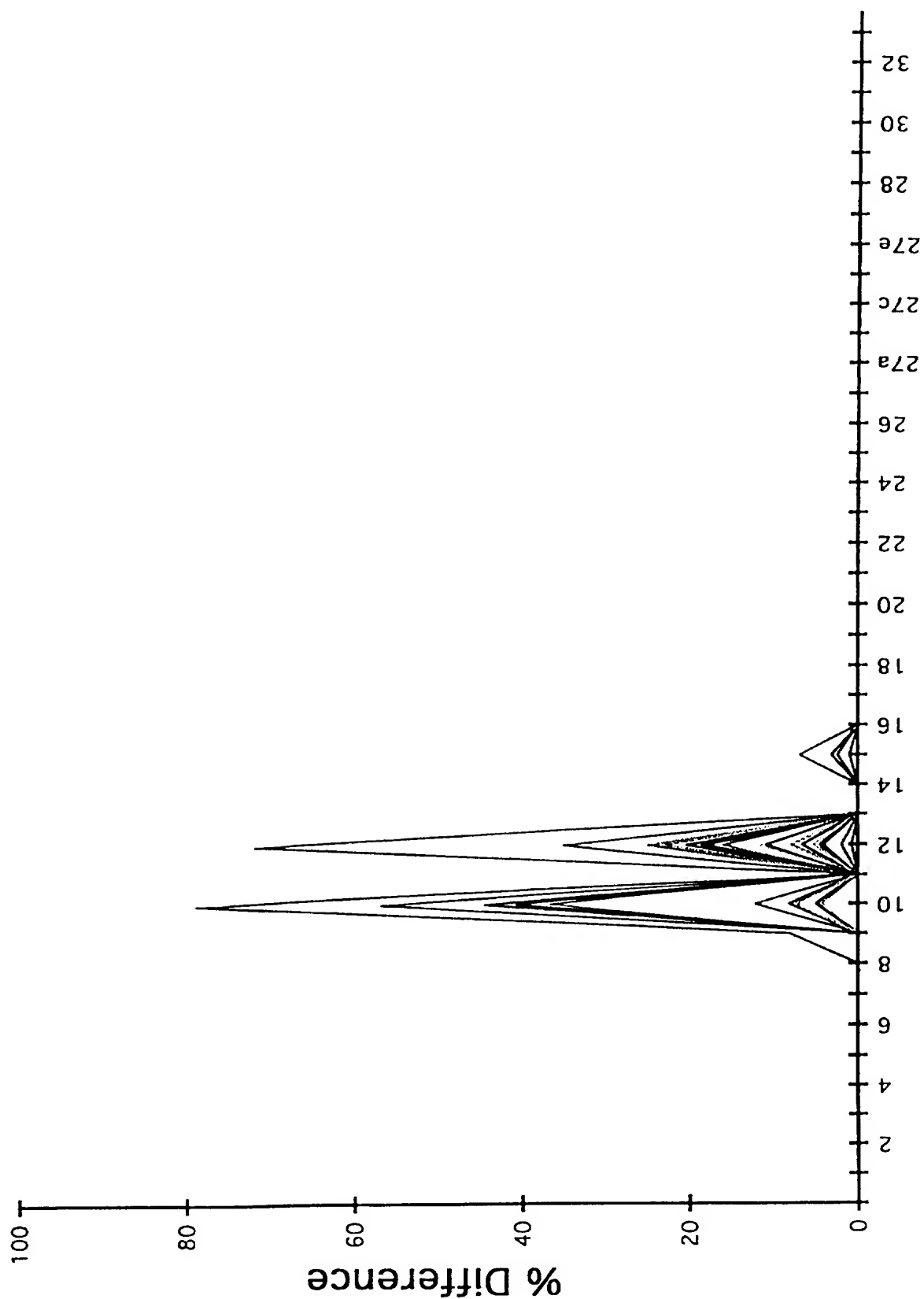
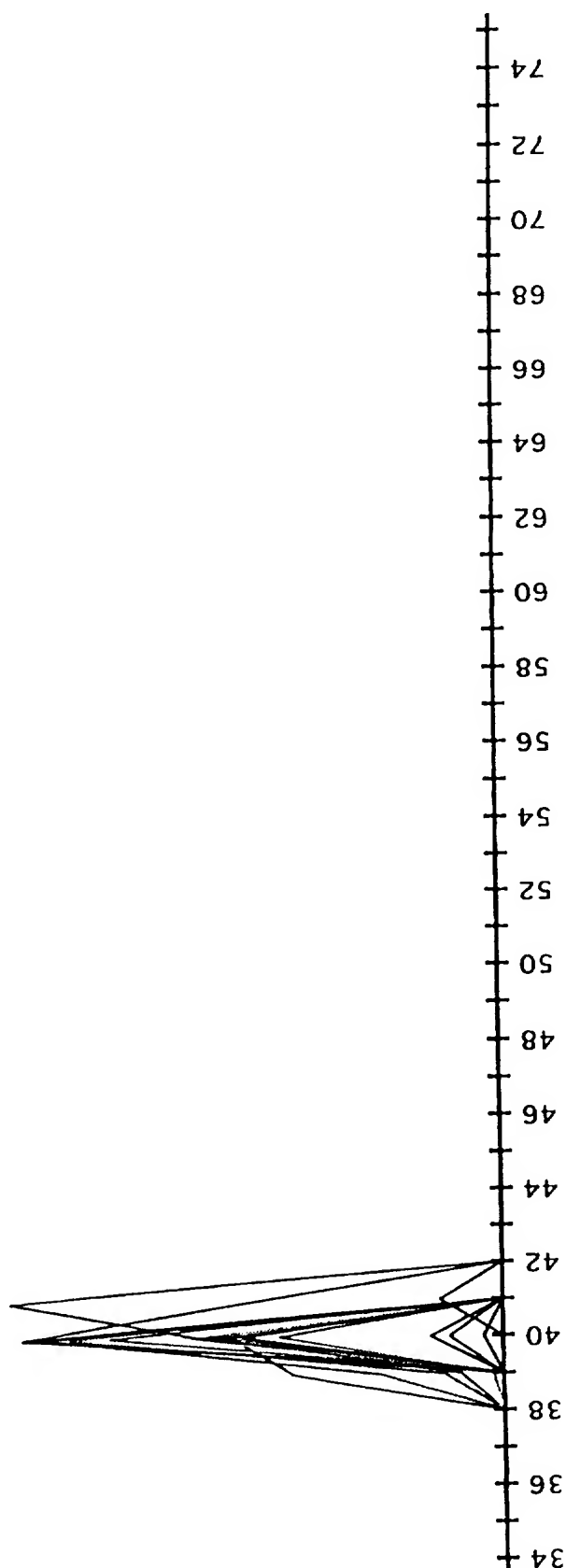


Figure 2a: Variable/constant domain interface residues for VL



**Figure 2a: Variable/constant domain interface residues for VL  
(cont.)**



**Figure 2a: Variable/constant domain interface residues for VL  
(cont.)**

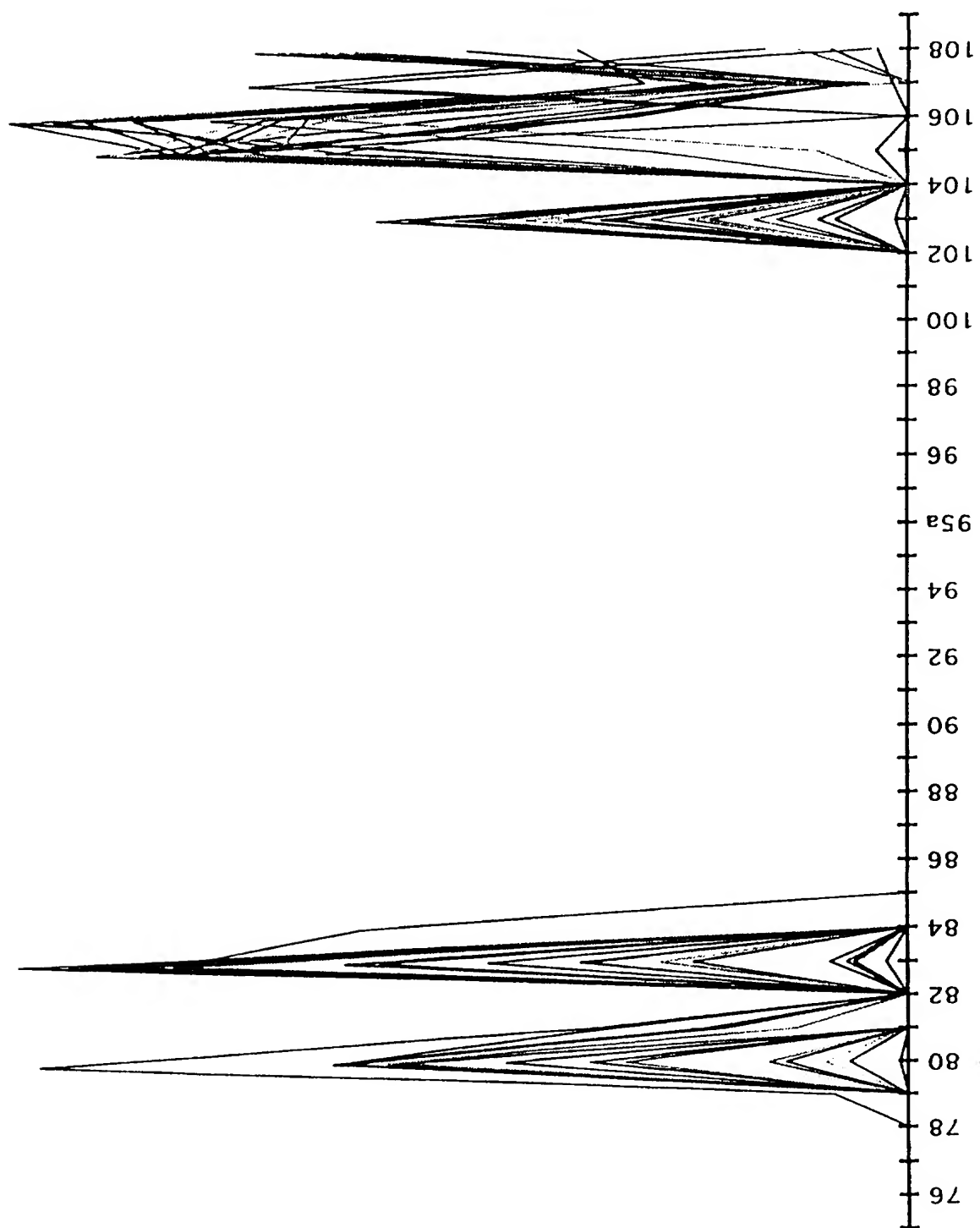






Figure 2a: Variable/constant domain interface residues for VL  
(cont.)

41	○	G	G	G	G	G	G	G	G	G	G	D	G	G	D	G	G	G	G	D	G	G	G	G	H	G	D	G
40	○	■	●	■	■	●	▲	■	■	▲	●	■	■	■	■	■	■	■	■	▲	■	■	▲	▲	■	■	■	■
39	○	■										●												●		●		●
38		K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K
37		Q	Q	Q	Q	Q	Q	Q	Q	Q	E	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q
36		Q	Q	Q	L	Q	Q	Q	H	Q	Q	Q	L	Q	L	Q	Q	Q	L	L	Q	Q	Q	L	Q	Q	L	Q
35		Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
34		W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
33		H	Y	A	H	A	A	H	T	A	H	N	T	N	N	E	N	S	V	H	H	N	N	N	Y	E	A	H
32		M	Y	L	L	L	L	L	L	V	L	A	L	L	A	L	L	V	V	L	L	L	V	L	M	L	L	L
31		F	Y	N	Y	W	Y	Y	F	A	F	Y	Y	Y	Y	Y	Y	A	Y	Y	Y	T	S	Y	Y	F	N	
30		S	Y	S	T	R	N	T	N	T	S	N	T	N	T	N	T	N	T	T	T	N	S	S	N	T	N	T
29		K	Y	Y	N	S	H	S	K	N	N	S	K	N	S	N	Y	Y	S	N	N	S	S	V	V	D	K	
28		G	S	I	G	I	I	S	R	V	G	T	Q	G	T	G	I	G	V	G	G	I	G	S	S	G	I	
27f		Y	N	N	S	N	V	K	D	D	T	K	N	T	N	D	G	D	N	N	D	I			D	N	S	
27e		S																										
27d																												
27c																												
27b																												
27a																												
26		S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
25		A	A	A	S	A	A	A	S	A	A	S	S	S	S	S	A	G	A	P	S	A	G	A	A	S	A	

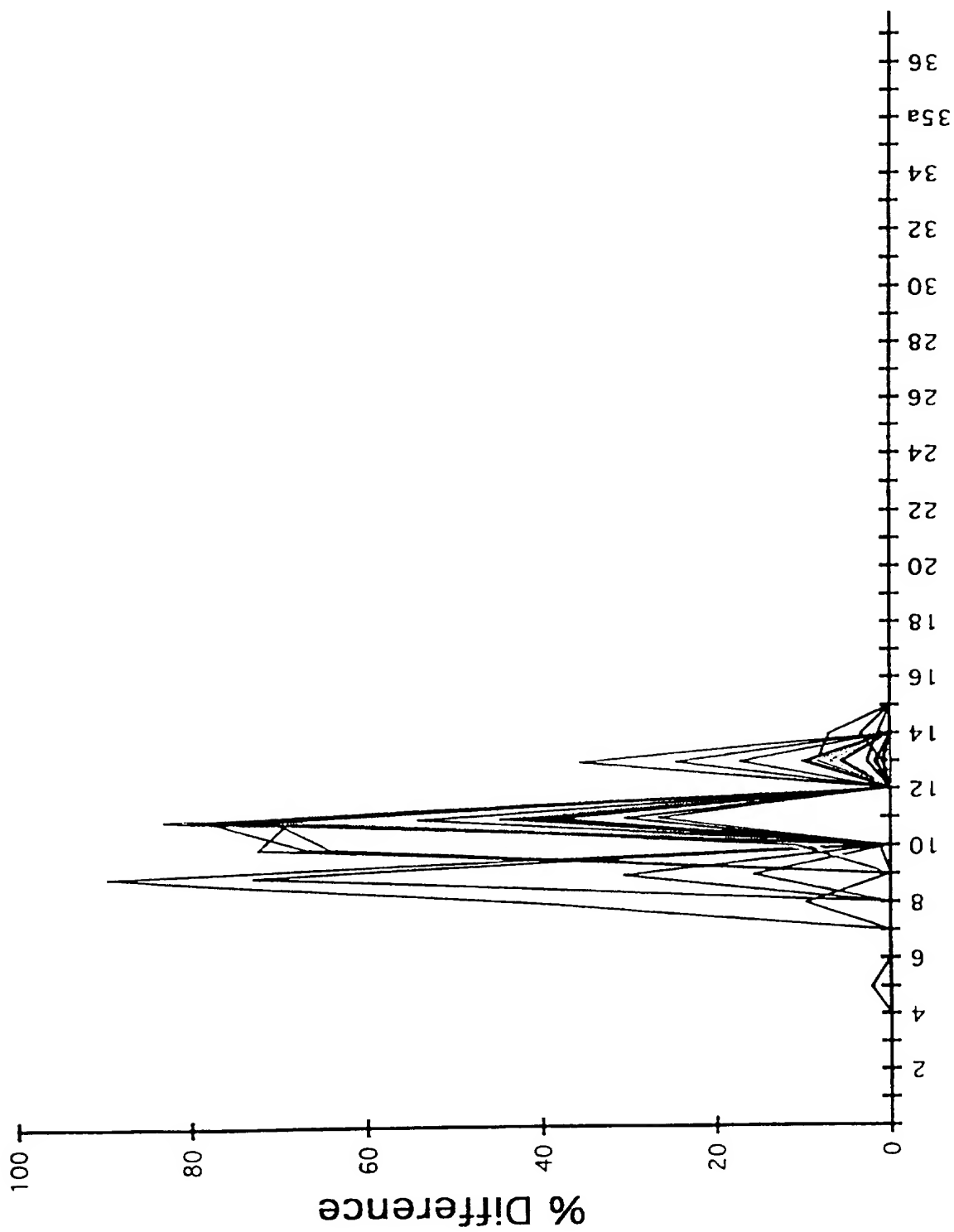


Figure 2a: Variable/constant domain interface residues for VL  
(cont.)

91	N	W	F	S	Y	F	Y	D	H	S	W	D	T	W	G	G	Y	H	G	S	G	W	W	G	D	S	S	G	W		
90	Q	Q	H	Q	Q	H	Q	N	Q	Q	L	N	Q	L	Q	Q	S	Q	Q	Q	Q	A	Q	Q	Q	N	Q	Q	Q	A	
89	Q	Q	Q	S	Q	Q	Q	Q	Q	Q	A	Q	S	A	E	Q	S	Q	S	S	Q	A	Q	Q	F	Q	Q	S	Q	Q	
88	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C		
87	Y	Y	Y	F	Y	Y	Y	Y	Y	Y	F	Y	F	F	Y	I	Y	Y	F	F	F	Y	Y	Y	Y	Y	F	F	F	Y	
86	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	
85	T	T	S	V	T	S	T	I	T	T	I	V	I	R	V	T	D	L	V	I	T	D	I	E	V	V	M	V	T	D	
84	A	A	G	G	A	G	A	A	A	A	A	A	G	A	G	A	A	A	G	G	A	T	A	A	G	A	G	G	A	A	
83	◆	●	◆	●		◆	●	▶	■	▲	●		▶	▶	◆		◆	◆	●	■	◆	■	◆	◆	◆	▲	■	■	■		
82	A	A	F	L	F	F	A	L	F	V	E	L	L	E	L	M	E	L	L	L	L	I	E	A	A	L	L	F	L	I	E
81	■	■					■										■					●			■						
80	▶	▶					◆	●	■						●	●	■	▲	●	●	▲	■	●	▲							
79	A	A	S	A	P	P	A	A	P	A	T	A	T	A	Q	A	A	A	A	H	S	A	T	A	A	T	A	Q	A	A	
78	E	E	Q	E	Q	Q	E	Q	Q	E	Q	Q	E	Q	Q	N	Q	Q	E	E	E	Q	E	E	E	Q	E	E	E	Q	
77	V	M	L	V	L	L	V	V	L	V	A	V	V	A	V	L	L	V	V	V	L	L	M	V	V	V	V	V	L	V	
76	P	R	S	R	S	S	S	S	S	P	G	S	R	G	R	N	G	S	R	R	N	G	T	S	R	S	S	R	N	G	
75	D	S	N	S	S	N	S	T	S	N	T	S	S	T	S	S	S	S	S	S	S	S	G	N	S	S	S	N	S	S	
74	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	
73	T	T	K	K	T	K	T	T	T	T	T	T	T	K	T	K	T	T	T	K	K	T	A	T	T	K	T	S	K	T	T
72	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	
71	T	S	S	T	T	S	S	T	T	T	A	T	T	A	T	S	S	T	T	T	T	S	S	S	S	T	T	T	T	T	
70	F	Y	Y	F	F	Y	Y	F	F	F	A	F	F	A	F	Y	A	Y	F	F	Y	A	Y	Y	F	F	F	F	Y	V	
69	D	S	Q	D	E	Q	S	D	D	D	K	D	D	K	D	D	T	D	A	D	D	S	S	S	D	D	D	D	D	T	
68	T	T	T	T	T	T	T	T	T	T	D	T	T	D	T	T	N	T	T	T	T	T	A	T	T	T	T	T	T	T	T



Figure 2b: Variable/constant domain interface residues for VH



**Figure 2b: Variable/constant domain interface residues for VH  
(cont.)**

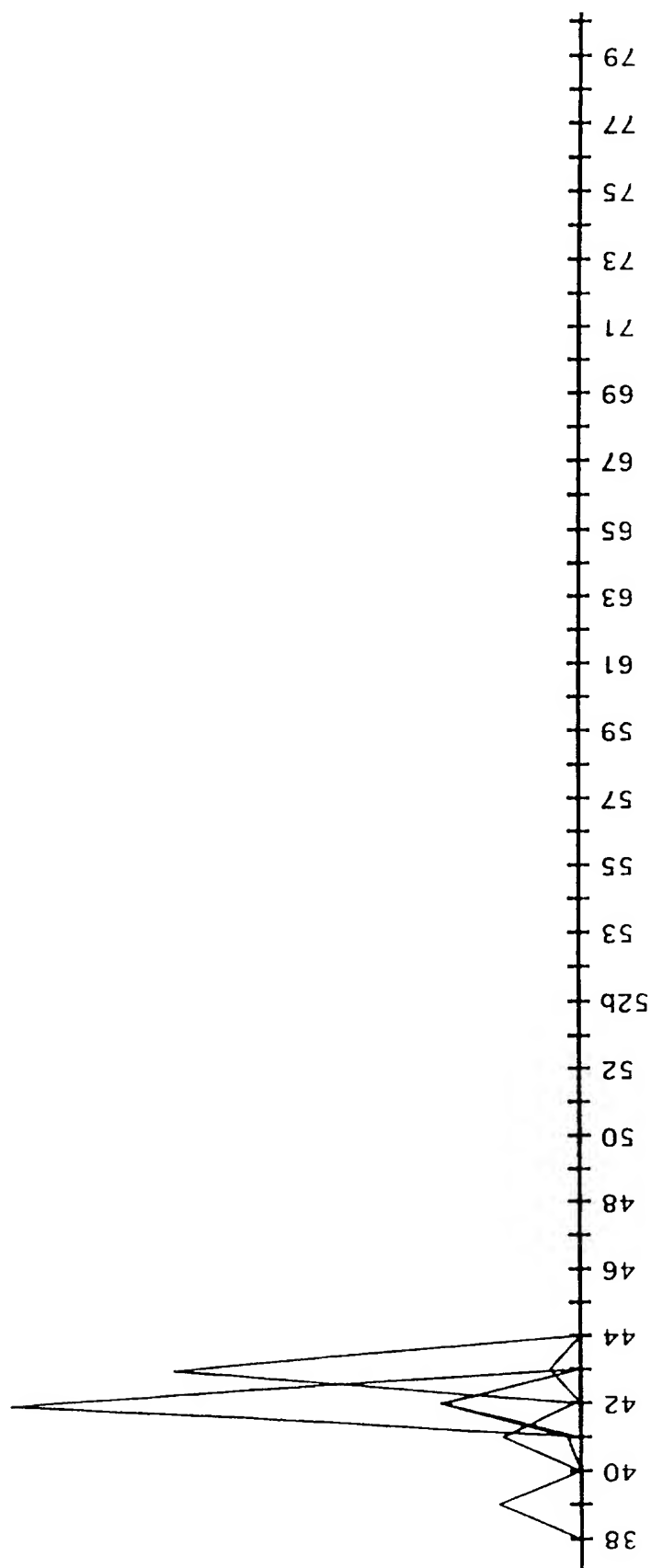
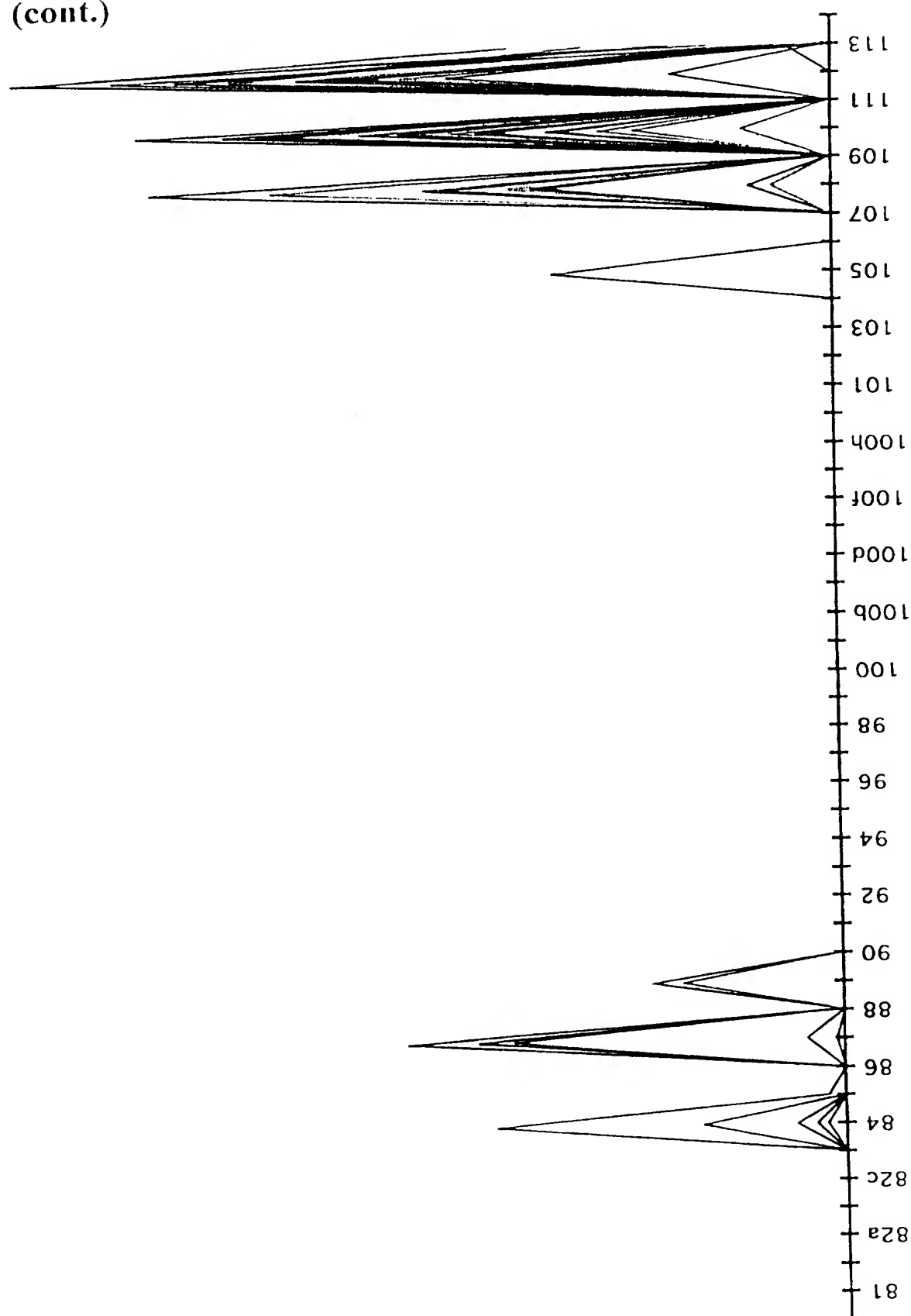


Figure 2b: Variable/constant domain interface residues for VII  
(cont.)





[illegible]

Figure 2b: Variable/constant domain interface residues for VH  
(cont.)

44	G	K	G	G	G	S	R	G	G	G	R	S	R	S	A	G	G	G	E	G	G	G	R	R	G	G
43	K	N	Q	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K
42	G	G	E	G	G	G	G	D	G	G	G	D	G	E	A	G	G	G	G	G	G	G	E	G	G	G
41	P	P	P	P	P	P	N	P	P	S	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
40	A	F	K	A	A	P	R	T	A	P	P	T	S	T	S	P	P	A	R	A	R	A	A	R	T	P
39	Q	Q	Q	Q	Q	Q	H	Q	Q	Q	Q	Q	Q	Q	Q	E	E	Q	Q	Q	Q	Q	K	Q	Q	Q
38	R	R	K	R	R	R	V	R	R	R	R	R	R	R	K	R	R	K	K	K	K	R	R	K	R	R
37	I	I	A	V	V	V	I	V	V	I	V	V	V	V	V	I	V	V	V	V	V	V	V	I	V	V
36	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W
35b	H	N	H	N	H	N	N	S	H	S	H	S	N	S	H	S	N	E	N	N	Y	S	E	S	E	S
35a	W	W	I	M	M	V	I	M	I	V	V	M	M	M	M	W	M	I	V	V	M	M	I	M	M	M
35	C	A	A	A	A	G	N	G	Y	G	G	G	Y	T	A	Y	Y	G	W	G	A	W	W	A	Y	Y
34	Y	Y	H	N	Y	Y	H	F	T	M	N	Y	F	E	Y	Y	Y	Y	Y	Y	Y	Y	Y	C	F	D
33	N	D	D	T	D	G	G	S	D	G	S	S	D	G	T	D	L	N	E	N	S	S	K	D	R	D
32	T	S																								
31	R																									
30	T	T	T	N	N	T	P	S	K	S	I	S	T	S	T	T	N	T	S	T	T	S	S	S	S	S
29	I	I	F	F	F	L	L	F	I	L	L	F	F	L	F	F	I	F	F	F	F	F	F	F	I	F
28	S	S	T	S	T	S	S	T	N	S	L	S	I	T	T	T	S	T	T	A	T	I	D	T	T	T
27	F	Y	Y	F	F	F	F	F	F	F	F	F	Y	F	Y	F	D	Y	Y	Y	Y	F	F	Y	F	
26	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	
25	S	T	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
24	V	V	A	A	A	V	A	A	A	F	V	A	S	A	G	T	V	A	A	A	A	S	A	A	A	A
23	I	T	K	A	A	T	K	A	A	S	T	A	K	A	K	A	T	K	K	K	K	S	A	K	A	S

Figure 2b: Variable/constant domain interface residues for VH  
(cont.)

45	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
46	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	D	E	E	E	E	E	E	E	E	E	E	E	E	E	E
47	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	Y	W	W	W	W	W	W	W	W	W	W	W	W	W	W
48	M	M	I	V	V	L	I	V	V	L	V	I	V	I	L	I	M	I	M	I	V	I	V	I	M	V	I	V		
49	G	G	G	A	S	G	G	A	A	A	G	A	G	A	G	G	G	G	G	G	A	G	G	A	A	G	A	G	A	
50	R	Y	Y	R	G	M	N	T	R	H	V	T	Y	T	L	F	Y	W	E	W	Y	I	E	E	G	A	Y	Q	Y	V
51	I	M	I	I	I	I	I	I	I	I	I	I	I	I	T	I	I	I	I	I	I	I	I	I	I	S	V	I	N	I
52	C	S	S	R	S	W	D	S	Y	F	W	S	S	L	S	R	Y	N	L	N	N	W	H	L	S	R	S	R	N	W
52a	Y	Y	P	S	W	G	P	N	P	W	A	N	P	S	P	N	Y	T	P	I	P	D	P	P	S	N	Y	N	P	Y
52b	E	S	G	K	D	D	Y	G	T	D	G	G	Y	G	S	K	S	N	G	Y	G	D	D	G	G	K	S	K	G	N
52c																														
53																														
54																														
55	G	G	D	Y	S	G	G	G	G	G	G	G	G	G	G	Y	G	G	G	G	G	S	G	G	S	Y	G	Y	G	S
56	S	S	D	A	S	N	G	Y	Y	D	N	Y	V	F	Y	T	S	E	R	E	Y	D	T	S	Y	T	S	E	Y	R
57	I	T	I	T	I	T	T	T	K	T	T	T	T	T	T	T	P	T	P	L	Q	I	T	T	T	T	T	I	T	
58	Y	R	K	Y	G	D	N	Y	R	R	N	Y	G	F	S	E	Y	T	N	T	S	H	N	N	F	E	Y	Y	A	Y
59	Y	Y	Y	Y	Y	F	Y	Y	Y	Y	Y	Y	Y	Y	Y	G	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	
60	S	N	N	A	A	N	N	Q	A	N	N	P	N	S	N	S	N	G	R	V	N	A	T	H	P	S	N	S	N	G
61	P	P	E	D	S	P	D	D	P	S	D	Q	A	G	A	P	E	E	D	E	D	P	E	D	A	P	D	E	D	
62	S	S	K	S	S	A	K	S	S	S	A	S	K	S	E	S	S	E	K	D	K	S	S	R	T	S	S	S	K	S
63	I	L	F	V	V	L	F	V	V	L	L	V	F	V	F	V	L	F	F	F	F	V	L	F	V	V	L	V	F	V
64	K	R	K	K	K	K	K	K	K	M	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K
65	S	S	G	D	G	S	G	G	G	S	S	G	G	G	G	S	G	G	G	G	D	G	G	S	G	G	G			
66	R	R	K	R	R	R	K	R	R	R	R	K	R	K	R	R	R	K	R	K	K	R	R	R	R	R	R	K	R	
67	S	I	A	F	F	L	A	F	F	L	V	F	A	F	A	F	V	F	A	F	T	F	F	A	F	F	I	F	T	F

Figure 2b: Variable/constant domain interface residues for VH  
(cont.)

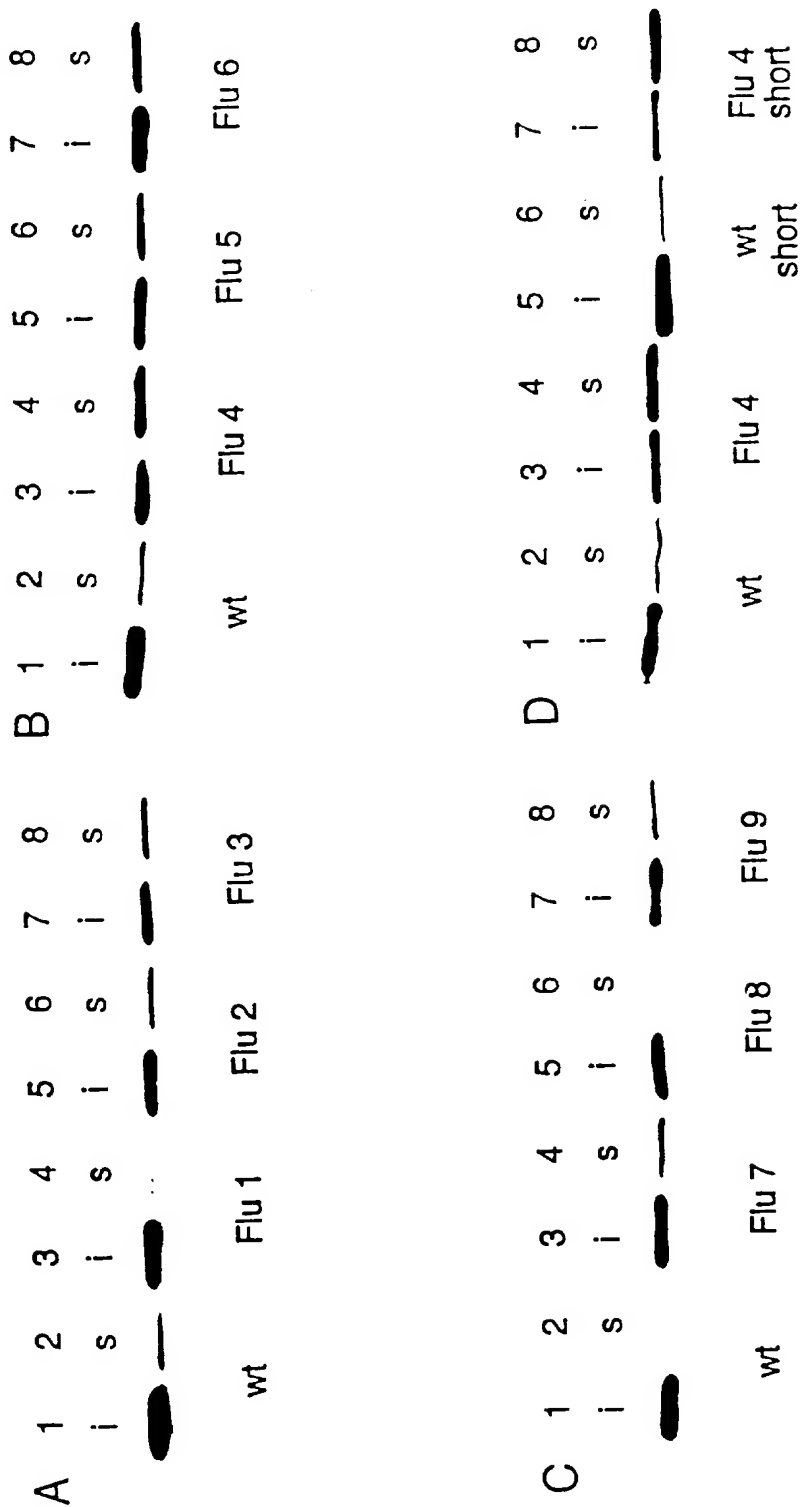
88	A A A A A A A G A A A A A A A A A A A A A G A A A A A G A A
87	T T S T M T S A T T T S S T S S T T S T A T T S T T T M S T
86	D D
85	E E E E E D E E E A D E E E E E A E E E E E E E E E E E E E E
84	N T S T A T S S A T T S S S S A A N S N S P S S S A T V S T
83	T T T K R H Q K R D Q K T R T R T K T K T R R T R R T R T R
82c	V V L L L L L L L V L L L L L L V L L L L L V L L L V L L L
82b	S S S N S S S S S S S S S S S R T S N S N S S K S S A S N S S
82a	I K N N N N T T N T K S R N A N S N S N R D S N S N N R N
82	L L L M M M L M M I M M L L L M L I L I L M M L M M L M L M
81	Q Q Q Q Q K H E Q K K Q E Q E Q K Q Q E Q Q Q Q Q D Q Q Q
80	I L M L L L M L L L L L M L M L S L M L M L L M L L L M M
79	F F Y Y Y F Y F Y F Y Y Y Y Y Y N Y Y Y F Y Y S Y Y Y Y Y
78	F F A L L V L L L V V L A L A L F A A A A L L A L L Y V A L
77	K Q T M S Q T T T Q Q T T N T I Q T T T T S T T T I Q S T T
76	N N S N N S S N N N S N S N S S N S N S S N N S N S N S S R
75	L K S Q K K S K K N K K S Q S Q K A S A S K K S R Q K K S K
74	S S S S A S S A S S S A S A S S S S S S S S S A S A S S S S
73	T T K D N N K N T T N N K N K N T T T T R D N T N T T D K N
72	D D D D D D D D D D D D D D D N E D E D N D D N D D D D D
71	R R A R R K V R A K K R V R V R V L A L V R R A R R R R V R
70	S T T S S S T S S S S S T S T S S S T S T S S T S S T S T S
69	I I L I I I L I I I I I L I M I I F F F L I I F I V I I L I
68	T S T T T S T T T K S T T T T T T A T A T T I T I I S T T T



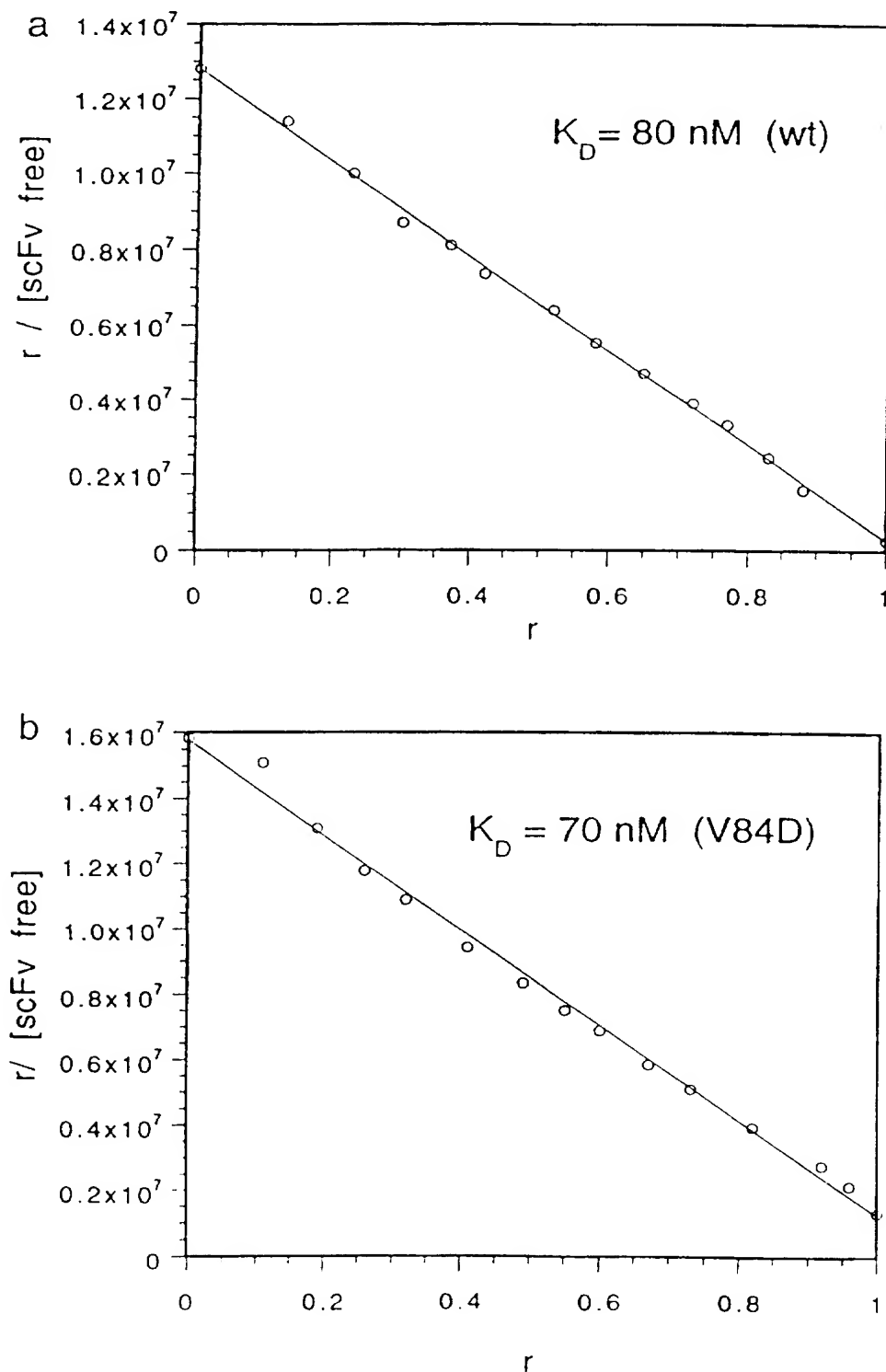
Figure 2b: Variable/constant domain interface residues for VH  
(cont.)

100i	F	L	Y	F	F	L	F	F	M	Y	M	F	M	F	F	A	V	S	M	F	F	P	N	F	F	F	G	M	F	F		
101	D	A	G	A	D	D	T	A	D	I	D	A	D	V	D	A	N	D	D	D	D	D	A	D	D	D	D	D	D	D		
102	V	Y	H	Y	I	Y	Y	Y	V	Y	Y	Y	Y	H	V	Y	P	Y	Y	V	S	Y	Y	G	Y	V	Y	Y	Y	Y		
103	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W		
104	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G		
105	Q	Q	Q	Q	Q	Q	R	Q	Q	Q	Q	H	H	A	Q	Q	Q	Q	A	Q	Q	Q	Q	A	Q	Q	Q	Q	Q	Q		
106	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G		
107	T	T	T	T	T	T	T	T	T	T	T	T	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	V		
108	T	Q	T	L	M	T	L	L	L	S	S	L	S	L	T	L	L	T	S	T	P	L	T	T	T	L	S	T	L	L		
109	V	V	L	V	V	L	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	L	V	V	L	L	V	V	L	V
110	T	S	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	
111	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	
112	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
113	S	E	S	A	S	S	A	A	S	S	S	A	S	A	S	A	S	S	A	S	S	S	A	S	S	S	A	S	S	S	S	

Figure 3: Western blots showing the insoluble (i) and soluble (s) fractions of cell extracts

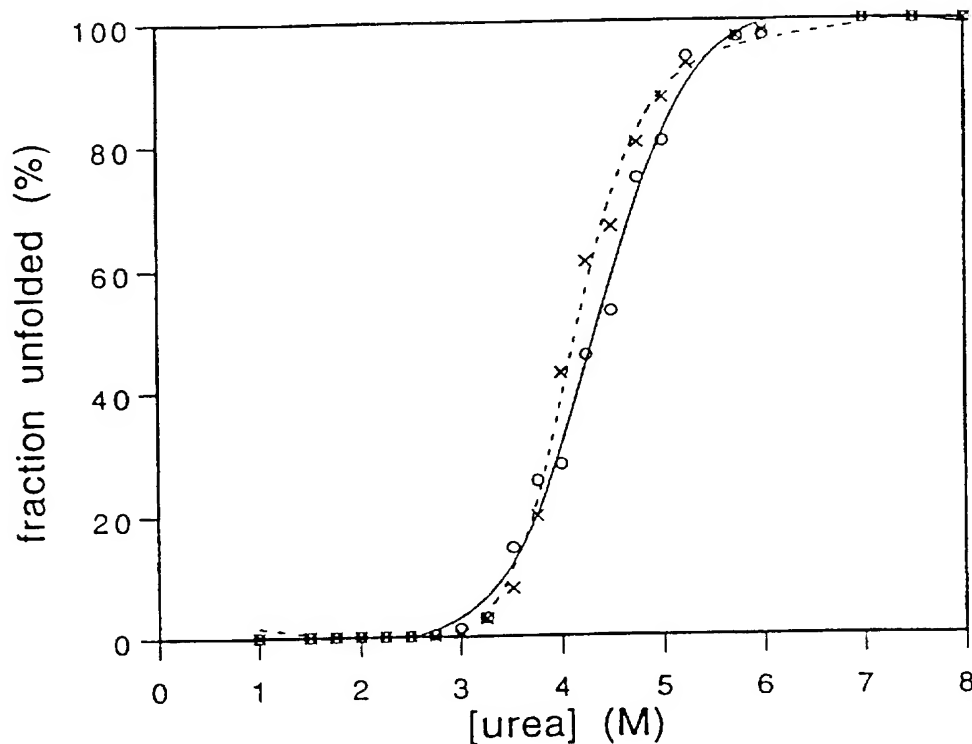


**Figure 4: Scatchard plots of fluorescence titration of fluorescein with antibody: a) Titration of wt scFv; b) Titration of Flu4(V84D)**





**Figure 5: Overlay plot of urea denaturation. (x) wt scFv, (o) Flu4**



**Figure 6: Thermal denaturation time courses at 40°C and 44°C for wt and Flu4 scFv fragments**

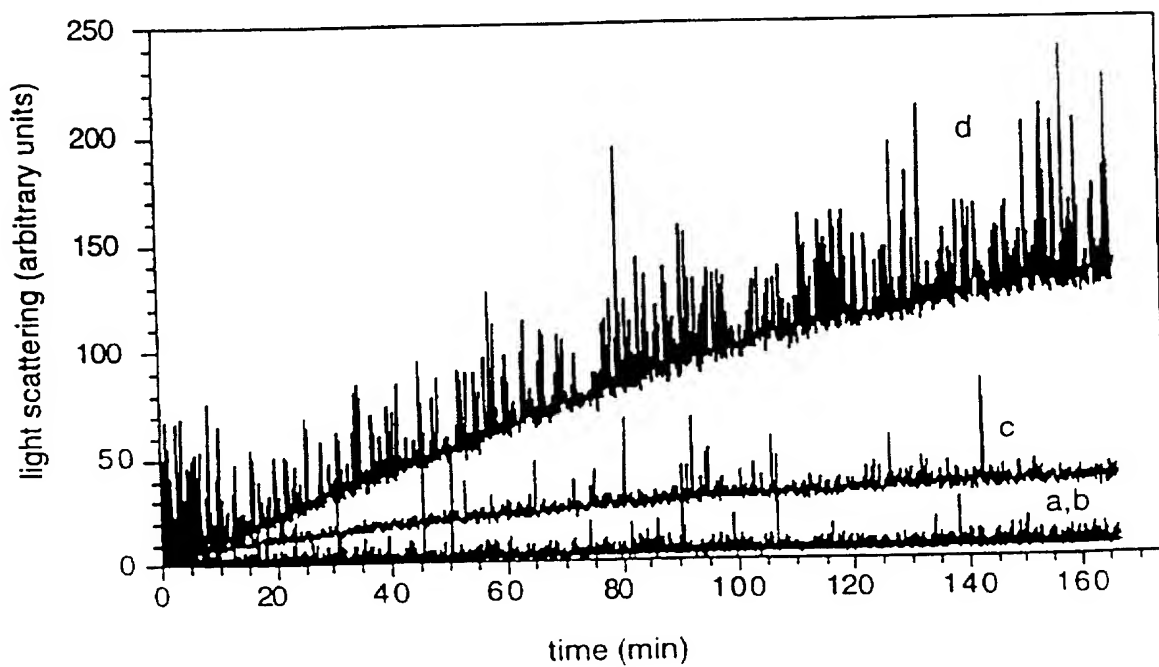


Table 1: Sequence variability of residues contributing to the v/c interface

Position	L9			L10			L12			L15			L39		
Species	L9			L10			L12			L15			L39		
Seq. 4-4-20															
Consensus															
Distribution:															
Asp	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Glu	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Lys	0	6	0	0	0	0	0	0	0	0	0	0	83	92	30
Arg	0	0	0	0	0	0	0	0	0	0	0	0	14	7	11
His	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20
Thr	0	4	0	1	49	6	3	0	93	1	0	0	1	0	1
Ser	43	24	90	4	48	60	84	50	99	0	1	0	1	0	0
Asn	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Gln	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
Gly	25	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Ala	11	41	7	90	0	0	4	23	0	0	8	1	0	0	0
Cys	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pro	0	2	0	0	0	0	11	20	0	45	28	84	96	0	0
Val	1	0	2	1	0	0	0	0	0	41	10	0	0	0	3
Ile	0	0	0	3	0	24	0	0	1	2	2	0	0	0	0
Leu	11	19	0	0	0	2	0	0	0	11	49	14	4	0	24
Met	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phe	0	1	0	0	2	7	0	0	0	0	1	0	0	0	3
Tyr	0	0	0	0	0	1	0	0	0	0	0	0	0	0	2
Trp	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Position

% exp. (FAB)

% exp. (ind.)

% buried v/c

Species

Seq. 4-4-20

Consensus

Distribution:

Table 1: Sequence variability of residues contributing to the v/c interface (cont.)

L40				L41				L80				L81				L83				L103			
64				114				57				70				17				42			
82				121				74				74				38				58			
22				3				21				5				46				26			
kappa		lambda		kappa		lambda		kappa		lambda		kappa		lambda		kappa		lambda		kappa			
hu	mu	hu	mu	hu	mu	hu	mu	hu	mu	hu	mu	hu	mu	hu	mu	hu	mu	hu	mu	hu	mu		
Pro				Gly				Ala				Glu				Leu				Lys			
Pro	Pro	Pro	Pro	Gly	Gly	Gly	Asp	Pro	Ala	Ala	Thr	Glu	Glu	Glu	Glu	Phe	Leu	Glu	Glu	Lys	Lys		
0	0	0	0	0	10	3	96	0	0	0	0	10	7	4	0	0	0	0	15	0	0		
0	0	0	0	0	4	0	0	0	8	0	0	86	93	69	82	0	0	99	68	0	0		
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	88	98		
1	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	8	1		
0	0	0	0	0	2	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
0	1	0	0	0	0	0	0	0	3	17	94	0	0	0	0	0	3	0	0	2	0		
2	19	0	0	0	1	0	0	9	6	19	0	0	0	0	0	2	0	0	0	0	0		
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
0	3	0	0	0	0	0	0	0	6	0	0	0	0	1	0	0	0	0	0	0	0		
0	0	0	0	0	80	90	3	0	0	0	0	1	0	17	0	0	1	0	0	0	0		
1	1	0	0	0	0	2	0	16	64	56	4	1	0	0	0	0	31	0	0	0	0		
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
94	77	92	96	0	0	0	0	74	9	2	1	0	0	0	0	0	0	0	0	0	0		
1	0	0	0	0	0	1	0	0	2	5	0	0	0	1	0	20	12	0	0	0	0		
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	11	0	0	0	0		
0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	32	0	0	0	0		
0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	2	0	0	0	0		
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	72	7	0	0	0	0		
0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0		
0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		

Table 1: Sequence variability of residues contributing to the v/c interface (cont.)

L105										L106										L106A										L107										L108									

**Table 1: Sequence variability of residues contributing to the v/c interface (cont.)**

H9			H10			H11			H13			H14			H41			H42			H43		
			65 72 8			31 71 58			69 73 5			36 36 0			72 72 0			111 115 3			65 78 2		
hu	mu		hu	mu		hu	mu		hu	mu		hu	mu		hu	mu		hu	mu		hu	mu	
Gly			Gly			Leu			Gln			Pro			Pro			Glu			Lys		
Gly	Pro		Gly	Glu		Leu	Leu		Lys	Lys		Pro	Pro		Ala	Arg		Pro	Pro		Gly	Gly	
0	0		5	2		0	0		0	0		0	0		0	0		0	0		0	2	
2	0		27	54		0	0		2	0		0	0		0	0		0	0		1	19	
0	0		0	0		0	0		59	54		0	0		0	3		0	0		0	0	
0	0		1	0		0	0		3	18		0	0		1	36		1	0		0	1	
0	0		0	0		0	0		1	0		0	0		1	0		0	11		0	0	
1	2		1	0		0	0		0	0		1	1		1	13		1	1		0	0	
1	1		0	2		0	0		0	0		3	3		10	18		2	3		8	0	
0	0		0	0		0	0		0	0		0	0		0	1		0	1		0	0	
0	0		0	0		0	0		34	22		0	0		0	0		1	0		0	0	
42	29		61	38		0	0		0	0		0	0		0	0		0	0		90	76	
33	31		3	1		0	0		0	5		1	8		59	7		1	0		1	2	
0	0		0	0		0	0		0	0		0	0		0	0		0	0		0	0	
21	36		0	0		0	0		0	0		96	85		18	14		94	83		1	0	
0	1		0	1		38	1		0	0		0	0		1	1		0	0		0	0	
0	0		0	0		0	2		0	0		0	0		0	1		0	0		0	0	
0	0		0	0		60	95		0	0		0	3		1	0		0	0		0	0	
0	0		0	0		0	0		0	0		0	0		7	0		0	0		0	0	
0	0		0	0		0	2		0	0		0	0		0	6		0	0		0	0	
0	0		0	0		0	0		0	0		0	0		0	0		0	0		0	0	
0	0		0	0		0	0		0	0		0	0		0	0		0	0		0	0	

Position

% exp. (FAB)

% exp. (ind.)

% buried v/c

Species

Seq. 4-4-20

Consensus

Distribution:

Asp

Glu

Lys

Arg

His

Thr

Ser

Asn

Gln

Gly

Ala

Cys

Pro

Val

Ile

Leu

Met

Phe

Tyr

Trp

Table 1: Sequence variability of residues contributing to the v/c interface (cont.)

H84		H87		H89		H105		H108		H110		H112		H113	
hu	mu	hu	mu	hu	mu	hu	mu	hu	mu	hu	mu	hu	mu	hu	mu
Val		Met		Ile		Gln		Ser		Thr		Ser		Ser	
Ala	Ser	Thr	Ser	Val	Val	Gln	Gln	Leu	Thr	Thr	Thr	Ser	Ser	Ser	Ser
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	10	0	1	0	0	0	0	0	0	0
0	0	0	0	1	0	5	0	3	0	0	0	0	0	0	0
0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
4	12	96	46	4	15	0	4	23	51	89	99	0	0	0	0
18	70	2	51	0	0	0	0	0	23	2	0	98	100	97	77
0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	80	83	0	0	0	0	0	0	0	0
55	12	0	1	0	0	0	11	0	0	0	0	0	0	0	22
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	1	0	0	0	0	2	0	1	0	0	0	1	0	2	0
4	0	0	0	76	50	0	0	0	0	0	0	1	0	0	0
1	1	0	0	5	13	0	0	0	0	0	0	0	0	0	0
0	0	0	0	6	7	0	0	63	25	0	0	0	0	0	0
0	0	0	0	7	13	0	0	9	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

**Table 2: Mutations introduced in the scFv fragment of the antibody 4-4-20**

	L15E (VL)	L11N (VH)	L11D (VH)	V84D (VH)
Flu 1	•			
Flu 2		•		
Flu 3			•	
Flu 4				•
Flu 5		•		•
Flu 6			•	•
Flu 7	•	•		
Flu 8	•		•	
Flu 9	•	•		•
Flu 4 short				•

**Table 3:  $K_D$  values of the different scFv mutants determined in fluorescence titration**

	Flu wt	Flu 3	Flu 4	Flu 6	Flu wt#
$K_D$ (nM)	$80 \pm 7$	$60 \pm 12$	$70 \pm 10$	$75 \pm 13$	90

# INTERNATIONAL SEARCH REPORT

In. ational Application No  
PCT/EP 97/03792

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K16/00 C12N15/12 C12N15/13 C12N15/63 G01N33/53  
A61K38/17 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 01787 A (CAMBRIDGE ANTIBODY TECH) 6 February 1992	1-16, 20, 25-34
Y	cited in the application see whole document, esp. page 3 ff.	21, 22
Y	--- HOPP T. P. ET AL.,: "A short polypeptide marker sequence useful for recombinant protein identification and purification" BIO/TECHNOLOGY, vol. 6, - October 1988 pages 1204-1210, XP002046115 cited in the application see the whole document --- -/--	21, 22



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

7 November 1997

Date of mailing of the international search report

24/11/1997

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Müller, F



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 97/03792

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MUYLDERMANS S ET AL: "SEQUENCE AND STRUCTURE OF VH DOMAIN FROM NATURALLY OCCURRING CAMEL HEAVY CHAIN IMMUNOGLOBULINS LACKING LIGHT CHAINS" PROTEIN ENGINEERING, vol. 7, no. 9, 1 September 1994, pages 1129-1135, XP000445081	1-12
Y	see abstract and p1130, 2.column, 7.para. -p1132	13,18, 19,26-34
Y	--- JENKINS T. M. ET AL.: "Catalytic domain of human immunodeficiency virus type 1 integrase: Identification of a soluble mutant by systematic replacement of hydrophobic residues" PROC. NATL. ACAD. SCI. USA, vol. 92, - June 1995, pages 6057-6061, XP002046116 see abstract and discussion	13,18, 19,26-34
A	--- BREITLING F. ET AL: "A surface expression vector for antibody screening" GENE, vol. 104, - 1991, pages 147-153, XP002046118 see the whole document	17
A	--- DALE J.E. ET AL.: "Improving protein solubility through rationally designed amino acid replacements: solubilization of the trimethoprim resistant type S1 dihydrofolate reductase" PROTEIN ENGINEERING, vol. 7, no. 7, - 1994, pages 933-939, XP002046117 see abstract, discussion p938, column 1; 1. paragraph	1-34
	-----	

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 97/03792

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9201787 A	06-02-92	AU 8238291 A	18-02-92
		CA 2087095 A	26-01-92
		EP 0540586 A	12-05-93
		JP 6502526 T	24-03-94
-----			



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 38/00, 38/55, C07K 14/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/05350</b> <b>(43) International Publication Date:</b> 12 February 1998 (12.02.98)
<b>(21) International Application Number:</b> PCT/US97/14005 <b>(22) International Filing Date:</b> 8 August 1997 (08.08.97)  <b>(30) Priority Data:</b> 08/689,528                      8 August 1996 (08.08.96)                      US  <b>(71) Applicant (for all designated States except US):</b> MILKHAUS LABORATORY, INC. [US/US]; Larry Hill Road, Corner of Westfall, R.D. 1 Delanson, Delanson, NY 12053 (US).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> MCMICHAEL, John [US/US]; Larry Hill Road, Corner of Westfall, R.D. 1 Delanson, Delanson, NY 12053 (US).  <b>(74) Agent:</b> SHARP, Jeffrey, S.; Marshall, O'Toole, Gerstein, Murray & Borun, 6300 Sears Tower, 233 South Wacker Drive, Chicago, IL 60606-6402 (US).		<b>(81) Designated States:</b> AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> MATERIALS AND METHODS FOR TREATMENT OF PLAQUING DISEASES  <b>(57) Abstract</b>  Methods and compositions are provided for alleviation of disease states involving plaque formation, such as are manifested in Alzheimer's Disease and other amyloid disorders, and arteriosclerotic disease. Methods for the treatment of herpes virus infections including chronic fatigue syndrome by administration of thimerosal are further provided by the invention.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

## MATERIALS AND METHODS FOR TREATMENT OF PLAQUING DISEASES

This is a continuation-in-part of U.S. Application Serial No.  
5 08/689,528 filed August 8, 1996 which is a continuation-in-part of U.S.  
Application Serial No. 08/249,175 filed May 25, 1994, which is a  
continuation-in-part of U.S. Application Serial No. 07/874,719, filed April 27,  
1992, which is a continuation-in-part of U.S. Patent Application Serial No.  
07/598,383, filed October 16, 1990, which is a continuation-in-part of U.S.  
10 Application Serial No. 07/514,021, filed April 27, 1990.

### FIELD OF THE INVENTION

The present invention relates to methods and materials for the  
treatment of diseases involving plaque formation including arteriosclerotic  
15 diseases and hypertension and more specifically to materials and methods for  
the treatment of atherosclerosis. As a further aspect of the present invention,  
materials and methods are provided for the treatment of herpes virus infections  
including but not limited to Herpes simples types 1 and 2, Epstein-Barr virus,  
cytomegalovirus, Herpes zoster and further for treatment of chronic fatigue  
20 syndrome

### BACKGROUND OF THE INVENTION

Cellular skeletal systems have three distinct ultrastructural  
features, microtubules, intermediate filaments, and microfilaments, all of  
25 which are fibrous macromolecules associated with the central nervous system  
(CNS). Neuronal intermediate filaments, defined as neurofilaments  
(containing amyloid beta protein constructs), are distinct from other  
intermediate filaments found in the cells of the central nervous system. R.D.  
Goldman, A. Milstead, J.A. Schloss and M.J. Yerna, *Annu. Rev. Physiol.*,  
30 41, p. 703-722 (1979); R.J. Lasak, *Neurosci. Res. Program Bull.*, 19, p. 7-32

(1981); R.J. Lasek and M.L. Shelanski, *Neurosci. Res. Program Bull.*, 19, p. 3-153 (1981); C.A. Mareta, ed., *Neurofilaments* (1983); M.L. Shelanski and R.K.H. Liem, *J. Neurochem.*, 33, p. 5-13 (1979). Neurofilaments are composed of three proteins with molecular weights of 200,000, 150,000 and 70,000 daltons. B.H. Toh, L.J. Gibbs, Jr., D.C. Gajdusek, J. Goudsmit and D. Dahl, *Proc. Natl. Acad. Sci. USA*. An additional 62,000 dalton protein is also affiliated with the above-mentioned proteins. Such proteins are associated with slow axoplasmic transport. P.N. Hoffman and R.J. Lasek, *J. Cell Biology*, 66, p. 351-366 (1975).

Alzheimer's Disease, and other amyloid associated maladies including senile dementia, Down's syndrome, Pick's disease, progressive supranuclear palsy, multiple sclerosis and others, are characterized by the presence of one or more fused fibrils of repetitive amyloid beta proteins or other similar amyloid residues such as paired helical filaments, neurofibrillary tangles, neuritic plaques, amyloid plaques and cerebrovascular amyloidosis. B.H. Anderson, D. Breinberg and M.J. Downes, *Nature*, 298, p. 84-86 (1982). These paired helical filaments are indistinguishable immunologically and chemically from normal neurofilaments and share many of the same proteinaceous epitopes. B.H. Anderson, D. Breinberg and M.J. Downes, *Nature*, 298, p. 84-86 (1982); B.H. Toh, L.J. Gibbs, D.C. Gajdusek, J. Goudsmit and D. Dahl, *Proc. Natl. Acad. Sci. USA*; K. Iqbal, I. Grundke-Iqbal, H.M. Wisniewski and R.D. Terry, *Brain Res.*, 142, p. 321-332 (1975). It has been suggested that they interfere with axonal transport. P.N. Hoffman and R.J. Lasek, *J. Cell. Biol.*, 66, p. 351-366 (1975); J.W. Griffin, P.N. Hoffman, A.W. Clark, P.T. Carroll and D.L. Price, *Science*, 202, p. 633-665 (1978).

Using a cDNA clone of the gene encoding amyloid beta protein as a genetic probe, it was shown that the gene is located on chromosome twenty-one and is expressed in many tissues of the body. D. Goldjager, M.I. Lerman, O.W. McBride, U. Suffiotti and D.C. Gaidusak, *Science*, 235, p.

77-780 (1987); R.E. Tanzi, J.F. Gusella, P.C. Watkins, G.A.P. Bruns, P. St. George, M.L. Vankeuren, D. Patterson, S. Pagan, D.M. Kurnit and R.L. Neve, *Science*, 235, p. 880-884 (1987). Quantitation of amyloid beta protein expression, as seen by its mRNA levels using the cDNA probe, has revealed  
5 that its level of expression in brain tissue of Alzheimer's patients was not above that seen for other tissues outside the central nervous system. Such a finding was of interest to researchers when noting that amyloid plaque formation only occurs in the brain. R.E. Tanzi, J.F. Gusella, P.C. Watkins, G.A.P. Bruns, P. St. George, M.L. Vankeuren, D. Patterson, S. Pagan, D.M.  
10 Kurnit and R.L. Neve, *Science*, 235, p. 880-884 (1987).

Amyloid beta protein is obtained through conventional means known in the art and has been characterized in various reports. A.S. Cohen and E. Calkins, *Nature*, 183, p. 1202 (1959); A.S. Cohen and E. Calkins, *J. Cell Biology*, 21, p. 481 (1964); A.S. Cohen, E. Calkins and C. Levens, *Am. J. Pathol.*, 35, p. 979 (1959). More recent work is manifested by D. Caspi, M.C. Baltz and M.K. Pepys, *Mol. Biol. Med.*, 3, pp. 387-407 (1986); and D. Caspi, M.C. Baltz and M.K. Pepys, *Mol. Biol. Med.*, 3, pp. 409-424 (1986). Amyloid beta protein exists in various structural forms. The amyloid beta protein that has been experimentally used and as referred to herein in terms  
15 of any specific embodiments constitutes a mixture of such forms. It is to be understood that within the scope of the present invention, it is contemplated that any of the various forms of amyloid beta protein may be used.

Amyloid beta protein from the brain has been cDNA cloned and shown to contain a unique twenty amino acid NH<sub>2</sub>-terminal sequence.  
25 Glenner, G.G. and Wong, W., *Biochem. Biophys. Res. Comm.*, 122, No. 3, pp. 1131-35 (1984); D. Caspi, M.C. Baltz and M.K. Pepys, *Mol. Biol. Med.*, 3, pp. 409-424 (1986); Goldgaber, D., Lerman, M.I., McBride, O.W., Saffiotti, U. and Gaidusak, D.C., *Science*, 235, pp. 777-80 (1987).

It has been observed that a buildup of abnormally organized  
30 amyloid beta protein in brain tissue is manifested in Alzheimer's Disease. See

Dennis J. Selkoe and Carmela R. Abraham, "Isolation of Paired Helical filaments and Amyloid Fibers from Human Brain," 134, *Methods in Immunology*, 388-404 (1986). The fact that there is an accumulation of beta amyloid protein in the brain in Alzheimer patients has been demonstrated by post mortem analysis of brain tissue that manifest a concentration of amyloid beta protein as part of an accumulation of parallel filaments or neural fibrillatory tangles in the brain that appear characteristic of Alzheimer victims, along with neuritic plaque and cerebral vasculatory amyloidosis.

The presence of amyloid beta protein in fibrils and plaques in Alzheimer's Disease, as well as other CNS disorders, has been suggested to be a result of a degradation product of the normal neurofilaments, D. Goldjaber, M.I. Lerman, O.W. McBride, U. Suffiotti and D.C. Gaidusak, *Science*, 235, p. 77-780 (1987); R.E. Tanzi, J.F. Gusella, P.C. Watkins, G.A.P. Bruns, P. St.George, M.L. Vankeuren, D. Patterson, S. Pagan, D.M. Kurnit and R.L. Neve, *Science*, 235, p. 880-884 (1987); M. Baudry, B.R. Dubrin, L. Beasley, M. Leon and G. Lynch, *Neurobiol. Aging*, 7, p. 255-260 (1986); G.G. Glenner, *Arch. Path. Lab. Med.*, 107, p. 218-282 (1983); or possibly due to improper metabolism of byproducts. Further breakdown products of amyloid beta proteins from neurofilaments have also been observed in amyloid plaques along meningeal vascular walls and intracortical blood vessels. S. Bahmanyar, E.J. Williams, F.B. Johnson, S. Young and D.C. Gaidusak, *J. Comp. Path*, 95, p. 1-5 (1985); M.E. Bruce and H. Fraser, *Neuropathol. Appl. Neurobiol*, 1, p. 189-207 (1981); M.E. Bruce and H. Fraser, *Neuropathol. Appl. Neurobiol.*, 7, p. 289-298 (1981); G.G. Glenner and W. Wong, J. Quaranta and G.G. Glenner, *Proc. Natl. Acad. Sci.*, 82, p. 8729 (1985); D.J. Selkoe, C.R. Abraham, M.B. Podlisky and L.K. Duffy, *J. Neurochem.*, 46, p. 1820 (1986).

During the mid-1960's, Solomon & Moos speculated that there was a close integration between immunological function, the central nervous system, psychophysiological factors (emotions), and disease, both physical and



mental. G.F. Solomon and R.H. Moos, *Arch. Gen. Psychiatry*, 11, p. 657-674 (1964). The integration of those systems was initially suggested through observation of the presence of abnormal immunoglobulins in schizophrenic patients. G.F. Solomon and R.H. Moos, *Arch. Gen. Psychiatry*, 11, p. 657-674 (1964); J.G. Knight, *Lancet*, 82, p. 1073-1076 (1982); W.J. Fessel and M. Hirata-Hibi, *Arch. Gen. Psychiatry*, 9, p. 601-613. These immune aberrations (termed autoantibodies), which seemed to target certain body cellular structures, G.F. Solomon, *Psychoneuroimmunology*, p. 259-278 (1985); G.F. Solomon and R.H. Moos, *Psychosom. Mod.*, 27, p. 135-149 (1981), supported the concept that there is a close communication between the CNS and the immune system. For instance, met-enkephalin is a neurotransmitter in the CNS and is a product of activated T-helper cells. G. Zurawaki, M. Benedik, D.J. Kamb, J.S. Abrams, S.M. Zurawaki and F.O. Lee, *Science*, 232, p. 772-775 (1986).

The appearance of autoantibodies specific to the CNS neurofilaments in patients with Alzheimer's and other CNS disorders suggests that the body's immune system may play a role in the disease process. S. Bahmanyar, R.K.H. Liem, J.W. Griffin and D.C. Gajdusek, *J. Neuropathol. Exp. Neurol.*, 53, p. 85-90 (1984); S. Bahmanyar, M.C. Moreau-Dubois, P. Brown, F. Catala and D.C. Gajdusek, *J. Neuroimmunol.*, 5, p. 191-196 (1983); T.S. Elizan, J. Casals and M.D. Yahr, *J. Neurol. Sci.*, 59, p. 341-347 (1983). The autoantibodies against normal CNS neurofilaments react with the paired helical filaments in neurofibrillary tangles characteristic of Alzheimer's Disease. D. Dahl and A. Bignami, *Exp. Neurol.*, 58, p. 74-80 (1978); M.E. Bruce, *J. Neuropathol. Exp. Neurol.*, 37, p. 595, abstract (1978).

Animal models for these CNS disorders, which are induced with aluminum chloride or B,B'-iminodipropionitrile (IDPN) to form paired helical filaments in neurofibrillary tangles, also react with antibodies directed

against CNS neurofilaments. J.W. Griffen, P.N. Hoffman, A.W. Clark, P.T. Carroll and D.L. Price, *Science*, 202, p. 633-665 (1978).

Control of such autoimmune reactions may lead to the alleviation of symptoms manifested by such reactions. Over the past two decades, a body of clinical literature has accumulated relating to the treatment of autoimmune disease (or, more appropriately, diseases reflecting immune dysfunction) using a technique called provocative-neutralization therapy. Miller, *Annals of Allergy*, 38, p. 185-191 (1977); Miller, *Trans. Am. Soc. Opth. & Otolar. Allergy*, 14, p. 159-168 (1974); Miller, *Clinical Medicine*, 81, p. 16-19 (1974). In short, this method, which is commonly employed for allergy therapy, involves subcutaneous or sublingual introduction of an antigen known, or suspected, to provoke symptoms reflective of immune dysregulation. By serial titration of the provoking material, a concentration of that agent may be determined which will neutralize those symptoms induced by the same substance at a different concentration. That is a prime example of a dose-dependent phenomenon in which one dose induces a positive reaction while another dose of the same agent induces a negative response.

Although it is thought that neutralization occurs as a consequence of reestablishing homeostatic functional levels of T8 suppressor cells, it is quite possible that the same antigen used at a neutralizing concentration to reverse immune dysregulation could also, or instead, trigger endocrine and/or neuronal control mechanisms to reverse symptoms. Because of the intimate association between the three control systems (endocrine, immune, nervous) and proven communication pathways between and among the cells comprising these respective systems, a single active molecule, such as amyloid beta protein in the Alzheimer's victim, and related CNS disorders, may reverse symptoms via any or all of these routes.

Plaque formation is a common component in the etiology of numerous other disease as well. Principal among those are arteriosclerotic diseases. Like Alzheimer's and related diseases, arteriosclerotic diseases,

such as atherosclerosis, are plaquing diseases. Such diseases are characterized by arterial plaque formation. These plaques commonly occur in large and medium-sized arteries and generally comprise cells, connective tissue (usually elastin, collagen, and glycosaminoglycans), and lipid deposits. The mixture of those components is usually complex, forming lesions which may be calcified in advanced stages of the disease. Plaque mass slowly increases throughout life, as blood vessels undergo progressive concentric fibromuscular thickening. In atherosclerotic patients, fibromuscular thickening of the intima of blood vessel walls proceeds rapidly and contributes, along with lipid deposition, to restricted blood flow. In non-atherosclerotic patients, the normal thickening of the walls of blood vessels does not contribute to increases in blood pressure and does not compromise blood flow. In fact, plaquing diseases often occur together and patients with neural plaques also have vascular plaques.

It is upon the matrix of fibromuscular thickenings that atherosclerotic plaques develop. Such plaques generally become more prevalent in the third decade of life, with localization being most common in the coronary arteries. Atherosclerotic lesions are generally thought to develop from fatty deposits which transiently occur in all humans in the developed muscular lining of blood vessels. The mechanism of transformation from fatty deposits or "streaks" to atherosclerotic lesions appears to be unknown. However, at least one report suggests that a virus may cause transformation of the normal lipid streaks to atherosclerotic plaques. Melnick, *et al.*, *JAMA*, 263: 2204-207 (1990); wherein it was reported that an avian herpesvirus stimulated atherosclerotic lesions in chickens. The above-cited authors also correlated the presence of cytomegalovirus in humans with atherosclerotic lesions in humans. A finding of herpesvirus and cytomegalovirus antigens, as well as nucleic acids encoding those viruses, in arterial smooth muscle suggests that viral infection of arterial cells may be coincident with the

development of atherosclerosis. However, a causative relationship between any virus and atherosclerosis has yet to be conclusively determined.

Of interest to the present invention is the chronic fatigue syndrome. Chronic fatigue syndrome is a disorder of which the major symptom is chronic, debilitating fatigue that is not resolved with bed rest, and which is severe enough to reduce daily activity below 50% for at least six months. In order to confirm diagnosis, eight of the following symptoms must have also begun at the onset of fatigue and have persisted or recurred over a period of at least six months. These symptoms include, mild fever, sore throat, painful lymph nodes, muscle weakness, muscle aches, fatigue after exercise, headaches, painful joints, neuropsychiatric complaints, sleep disturbances, and sudden onset in a healthy person. A diagnosis of chronic fatigue syndrome further involves elimination of a variety of other illnesses characterized by fatigue through personal history, physical examination and laboratory findings.

Chronic fatigue syndrome is a disorder that may have several causes. Much of the early literature on chronic fatigue syndrome focuses on the Epstein-Barr virus as a causative agent. The Epstein-Barr virus is a herpes-like virus that is the major cause of acute infectious mononucleosis, a common syndrome characterized by fever, sore throat, extreme fatigue, and swollen lymph glands.

It was later reported in the art that the rubella virus may have a possible role in the etiology of chronic fatigue syndrome. Studies conducted on patients having chronic fatigue syndrome have shown that many of those patients have abnormally high levels of antibody to the rubella virus.

The use of the influenza virus vaccine and the rubella virus vaccine both separately and together have been reported in the art for the treatment of herpes (Epstein-Barr virus) virus infections. Lieberman, *Clinical Ecology*, 7(3):51 (1990) reported the use of patients suffering from Epstein-Barr virus with influenza virus vaccine given together with histamine

and the immune enhancer Staphage lysate. Patients were also successfully treated with the same composition further in combination with rubella virus vaccine and with rubella virus vaccine alone.

Also of interest to the present invention is the disclosure of McMichael  
5 U.S. 4,521,405 that patients experiencing recurrent herpes simplex virus type II infection have reported relief of lesion pain and lesion enlargement upon treatment with compositions including histamine, measles inactivated, attenuated virus and influenza vaccine (killed) virus. McMichael, U.S. 4,880,626 taught a composition for alleviating the symptoms of AIDS  
10 comprising human chorionic gonadotropin, Staphage lysate, an influenza virus vaccine, such as Fluogen™ and fractionated inactivated HIV virus.

Also of interest to the present invention is hypertension. The increases in vascular permeability generally observed in hypertension may increase influx of lipoprotein into cells, thus increasing the likelihood of  
15 atheroma formation. Hypertension may also contribute to atherosclerosis in blood vessels surrounding the brain. A reduction in hypertension has been shown to significantly reduce the incidence of myocardial infarction associated with atherosclerosis. Other factors in the development and progression of atherosclerosis include diabetes mellitus, which may reduce lipid efflux from  
20 cells in the arterial wall. In addition, cigarette smoking dramatically increases the risk of developing atherosclerosis and associated hypertension, including their sequelae, such as infarction of the myocardium and brain. Obesity is another factor which may contribute, especially in an individual who smokes. Overall, hypertension is the single greatest risk factor in coronary diseases as  
25 well as cerebrovascular stroke.

The presence of hypertension is a primary indicator of an arteriosclerotic condition and is often used by physicians as the sole diagnostic measure of diseases such as atherosclerosis. Moreover, a reduction of blood pressure is thought to have an effect in reducing the severity of atheroma  
30 plaques. The mechanism for such reduction may be a reduction in the

transport of lipids and proteins into blood vessels which is coincident with a reduction in blood pressure. The diastolic component of blood pressure is generally thought to be the primary indicator of hypertension. While the systolic component may vary greatly depending upon nervousness, anxiety and the like, diastolic blood pressure generally remains constant and is more reflective of a patient's general vascular state. A diastolic reading of over 90 is considered mild hypertension in an adult and a diastolic reading of over 100 is considered hypertensive and an indicator of arteriosclerotic disease.

10

#### SUMMARY OF THE INVENTION

The present invention provides methods for alleviating the symptoms of disease states associated with plaque formation. In accordance with the invention, there is provided a method to stimulate the appropriate metabolic regulatory systems (immune, CNS or endocrine) which retard the progress of the symptoms of plaquing diseases, such as Alzheimer's and related diseases and arteriosclerotic diseases. Observations by scientists have now indicated that the apparent elevated amyloid beta protein concentration in, for example, Alzheimer's diseases may not be due to an increase in genomic expression, but possibly to activation of a mechanism that induces the reorganization of amyloid moieties from normal neurofilaments into paired helical filaments resulting in neurofibrillary tangles, neuritic plaques or amyloid plaques. D. Goldjaber, M.I. Lerman, O.W. McBride, U. Suffiotti and D.C. Gaidusak, *Science*, 235, p. 77-780 (1987); R.E. Tanzi, J.F. Gusella, P.C. Watkins, G.A.P. Bruns, P. St.George, M.L. Vankeuren, D. Patterson, S. Pagan, D.M. Kurnit and R.L. Neve, *Science*, 235, p. 880-884 (1987); M. Baudry, B.R. Dubrin, L. Beasley, M. Leon and G. Lynch, *Neurobiol. Aging*, 7, p. 255-260 (1986); G.G. Glenner, *Arch. Path. Lab. Med.*, 107, p. 218-282 (1983). The mechanisms of the present invention may result in triggering control processes that correct the rearrangement of

30

neurofilaments, alter abnormal amyloid protein formation including amyloid beta formation, and/or allow for clearing of axonal transport mechanisms. Similarly, regulatory control systems, as noted above, play a role in arteriosclerotic plaque formation, leading to arteriosclerotic diseases such as atherosclerosis. A significant common occurrence in patients having arteriosclerotic disease and/or neural plaquing disease, such as Alzheimer's, is hypertension. Accordingly, methods of the present invention cause a reduction in hypertension as an indication of alleviation of the overall disease state. The diseases susceptible to treatment with methods according to the invention have in common plaque formation. Accordingly, treatment with methods according to the invention provides an effective treatment of all such diseases by alleviating causative symptoms of the disease. In addition, as detailed below, compositions and methods of the invention are useful in the reduction of hypertension generally.

As one aspect of the invention, it has been discovered that a component of influenza virus vaccines that provided activity against herpes viruses as reported in Lieberman, *Clinical Ecology*, McMichael U.S. 4,521,405 and McMichael U.S. 4,880,626 was the preservative thimerosal which was present in commercially available influenza virus vaccines such as Fluogen™ (Parke Davis, Morris Plains, NJ), Fluzone™ (Connaught Laboratories, Swiftwater, PA), and Flu-Immune™ (Lederle, Wayne, NJ). Consequently, it is believed that results reported by McMichael and Lieberman attributed to the anti-herpes virus effects of influenza virus vaccine are due to the presence of thimerosal in the tested compositions. Further, it has been discovered that the anti-herpes virus activity of influenza virus vaccine is attributable to the presence of thimerosal in the tested vaccines and that herpes virus infections may be treated with thimerosal uncombined with or in the absence of influenza virus vaccine. Accordingly, the present invention provides methods for treating subjects suffering from herpes virus infections, comprising the step of administering an effective amount of a

composition comprising thimerosal free of association with influenza virus. More specifically, the present invention provides methods for treating patients having chronic fatigue syndrome comprising administering an effective amount of thimerosal free of association with influenza virus to a subject suffering from chronic fatigue syndrome. As one aspect of this invention it has been found that the combination of thimerosal free of association with influenza virus and rubella virus vaccine is particularly effective in the treatment of subjects suffering from chronic fatigue syndrome. The invention further provides methods for *in vitro* killing of herpes virus by administration of effective amounts of thimerosal. The invention further provides methods of treating plaquing diseases such as atherosclerosis and hypertension by combining thimerosal or thimerosal containing compositions such as influenza virus vaccines which contain thimerosal with amyloid beta protein.

In order to identify a dose of amyloid beta protein for use in the invention, a wheal produced upon intradermal injection of the therapeutic material was evaluated according to criteria set forth in Moore, *Clinical Medicine*, 81: 16-19 (1974), incorporated by reference herein. Upon subcutaneous injection, a wheal may be determined to be positive ten minutes after injection as a blanched, hard, raised, and discoid protrusion from the skin. A negative wheal is sufficiently absorbed at the end of ten minutes that the protrusion on the skin has grown less than an average of two millimeters in diameter from its original size.

In a preferred embodiment of the invention, compositions for treatment of plaquing diseases according to the invention comprise a dose from about  $10^{-10}$  to about  $10^{-2}$  mg of amyloid protein and from about 0.05  $\mu$ g to 500  $\mu$ g thimerosal with about 0.5  $\mu$ g to about 50  $\mu$ g thimerosal being preferred and about 5  $\mu$ g thimerosal being particularly preferred. The total volume of a typical composition according to the invention for administration to a patient is about 0.05 cc, or one drop. Compositions according to the



invention for treatment of plaquing diseases may comprise  $\beta$ -amyloid protein or the first 28 amino acids of  $\beta$ -amyloid protein.

Preferred dosages of thimerosal for treatment of subjects suffering from herpes virus infections range from about 0.05  $\mu\text{g}$  to 500  $\mu\text{g}$  thimerosal with about 0.5  $\mu\text{g}$  to about 50  $\mu\text{g}$  thimerosal being preferred and about 5  $\mu\text{g}$  thimerosal being particularly preferred.

Also in a preferred embodiment, compositions according to the invention are pharmaceutical compositions for treatment of arteriosclerotic diseases which pharmaceutical compositions comprise amyloid protein and thimerosal in a pharmaceutically-acceptable carrier.

Methods according to the present invention are useful in alleviating symptoms of arteriosclerosis generally, and atherosclerosis in particular. Such methods comprise the step of administering to a patient suspected or confirmed as having an arteriosclerotic disease an effective amount of a pharmaceutical composition comprising amyloid protein and an influenza virus vaccine. An effective amount of a composition according to the invention is an amount which results in a reduction in the symptoms of an arteriosclerotic disease. Most preferably, an effective amount of a composition according to the invention comprises from about  $10^{-10}$  to about  $10^{-2}$  mg of an amyloid protein, preferably  $\beta$ -amyloid protein, and about 0.05 and about 0.05  $\mu\text{g}$  to about 500 $\mu\text{g}$  thimerosal being preferred. An amyloid protein used in methods according to the invention may be a  $\beta$ -amyloid protein or may be the first 28 amino acids of a  $\beta$ -amyloid protein. A highly preferred amount of amyloid protein used in compositions and methods according to the invention includes from about  $10^{-5}$  and about  $10^{-2}$  mg of amyloid protein.

Methods and compositions according to the present invention are effective in alleviating symptoms of any disease in which plaquing is involved and especially diseases in which atheroma formation is characteristic. Compositions and methods according to the invention also alleviate hypertension and reduce cholesterol, both of which have a direct effect in

reducing the severity of or eliminating symptoms associated with arteriosclerotic disease. The following detailed description of the invention provides exemplification of claimed methods and compositions. However, it is understood by the skilled artisan that other uses of the invention, specifically relating to the treatment of arteriosclerotic diseases, are within the scope of the present claims.

Methods according to the present invention are useful in alleviating symptoms of arteriosclerosis generally, and atherosclerosis in particular. Such methods comprise the step of administering to a patient suspected or confirmed as having an arteriosclerotic disease an effective amount of a pharmaceutical composition comprising amyloid protein and an influenza virus vaccine. An effective amount of a composition according to the invention is an amount which results in a reduction in the symptoms of an arteriosclerotic disease. Most preferably, an effective amount of a composition according to the invention comprises from about  $10^{-10}$  to about  $10^{-2}$  mg of an amyloid protein, preferably  $\beta$ -amyloid protein, and about 0.05 and about 0.05  $\mu$ g to about 500 $\mu$ g thimerosal being preferred. Preferred dosages of thimerosal for treatment of subjects suffering from herpes virus infections range from about 0.05  $\mu$ g to 500  $\mu$ g thimerosal with about 0.5  $\mu$ g to about 50  $\mu$ g thimerosal being preferred and about 5  $\mu$ g thimerosal being particularly preferred. An amyloid protein used in methods according to the invention may be a  $\beta$ -amyloid protein or may be the first 28 amino acids of a  $\beta$ -amyloid protein. A highly preferred amount of amyloid protein used in compositions and methods according to the invention is from about  $10^{-5}$  and about  $10^{-2}$  mg of amyloid protein.

Methods and compositions according to the present invention are effective in alleviating symptoms of any disease in which plaquing is involved and especially diseases in which atheroma formation is characteristic. Compositions and methods according to the invention also alleviate hypertension and reduce cholesterol, both of which have a direct effect in

reducing the severity of or eliminating symptoms associated with arteriosclerotic disease. The following detailed description of the invention provides exemplification of claimed methods and compositions. However, it is understood by the skilled artisan that other uses of the invention, specifically relating to the treatment of arteriosclerotic diseases, are within the scope of the present claims.

Also in a preferred embodiment, the invention provides a method for alleviating the symptoms of disease states associated with abnormal accumulation of and/or molecular organization of amyloid protein or amyloid plaques, which comprises administration to a diseased patient of an effective amount of amyloid protein or an effective active fragment thereof. The amyloid protein is preferably an amyloid beta protein although amyloid protein fragments such as fragments comprising the first 28 amino acid residues of the amyloid beta protein are expected to be useful. The method of the invention is useful against disease states associated with abnormal accumulation of and/or molecular organization of amyloid protein or amyloid plaques including disease states in which the amyloid protein or plaques are associated with the central nervous system and histopathologically related disorders. Such diseases include, but are not limited to Alzheimer's Disease and Parkinson's Disease. Other disease states include those such as atherosclerosis.

## DETAILED DESCRIPTION OF THE INVENTION

### A. Application of Materials and Methods of the Invention to the Treatment of Alzheimer's and Related Diseases

The alleviation of Alzheimer's Disease symptoms observed following administration of amyloid beta protein as described herein likely reflects stimulation of appropriate metabolic regulatory systems in the Alzheimer's Disease patients such that accumulation and/or formation of the paired helical filaments in neurofibrillary tangle and/or amyloid plaque developments are significantly altered or slowed and accumulated proteins are

eliminated. This reprogramming to establish proper homeostasis would allow more efficient transmission of nerve impulses which would result in clinical improvement of treated Alzheimer's patients.

Typically, a pharmaceutical dosage unit of the present invention  
5 for the delivery of amyloid beta protein in a low concentration comprises a liquid or solid carrier and an effective amount of amyloid beta protein. One suitable carrier for sublingual administration comprises a phenylated saline solution. Effective amounts of the amyloid protein range from about  $10^{-10}$  to about  $10^{-2}$  mg, and preferably from about  $10^{-5}$  to about  $10^{-3}$  mg and most  
10 preferably about  $10^{-4}$  mg, amyloid beta protein in association with pharmaceutically acceptable excipients. The amyloid beta protein is administered through standard methods, including sublingual, subcutaneous and transdermal routes, and in dosage units that are either liquid or solid.

One explanation for the mode of action of this invention may  
15 be that the amount of this protein administered is sufficient to trigger a negative feedback mechanism to the body such that production of additional amyloid beta protein, possibly through breakdown of normal neurofilaments, is inhibited. Under this theory, the low level of amyloid beta protein, or a derivative thereof, gives a signal to the body to correct the abnormal  
20 synthesis/degradation process. The body sensors are then adjusted to normal metabolic control of amyloid beta protein processing that allows the proper balance to reestablish itself, alleviating the abnormal processing. The immune system, as well as the endocrine and CNS control systems, could play an integral regulatory role in response to the low dose therapy, with the amyloid  
25 protein functioning through mechanisms that not only correct the molecular organization of the amyloid beta protein moieties, but clear the interfering amyloid molecular constructs.

In a preferred embodiment, the present invention provides  
administration of amyloid beta protein or a derivative thereof. The amyloid  
30 beta protein may be provided either as part of a liquid solution or in a solid

powder matrix, and may be administered with conventional excipients to permit ease of administration and accurate dosage delivery. Patients characterized herein below were evaluated using a battery of objective tests designed to measure cognitive ability. These included the mini-mental State Examination, the Verbal Fluency Task Examination (word name task and category task), evaluation on the Demattis Dementia Rating scale, and the Word-Association Task Examination of the Wechster Memory Scale-Revised. Not all results of all tests are provided herein, however, the results of all the tests were qualitatively the same as those below and led to the same conclusions as those provided herein relative to the effect of treatment according to the invention.

#### EXAMPLE 1

A 67 year old male with a history of Alzheimer's Disease for four years prior to initiating therapy presented with an inability to answer questions, to place names with faces, and to complete his sentences. His wife noted a consistent downhill progression of his condition on a monthly basis. At the initiation of therapy, with the composition of the invention, his initial score on the Mini-Mental State Exam was 5 of a possible 30. The subject was treated by sublingual administration four times per day of a dosage unit comprising  $10^{-4}$  mg of amyloid beta protein in a phenylated saline solution. After five months of therapy according to the invention, the patient scored a 12 1/2 on the Mini-Mental State Exam, was reading road signs while travelling and was communicating with family members. Also, the patient appeared to be more relaxed and better able to respond to his wife's efforts to assist him.

#### EXAMPLE 2

An 81 year old male with a history of Alzheimer's Disease was treated according to the invention. Prior to treatment the subject was unable

to dress himself, had a flat affect, was poorly communicative and scored 10 1/2 on the Mini-Mental State Exam. The subject was treated by sublingual administration four times daily of the amyloid beta dosage unit of Example 1. After three months of treatment, the subject scored 17 points on the Mini-Mental State Exam, was more animated in speech, could dress himself most days and was more confident in physical actions.

### EXAMPLE 3

In this example, the subject was a 62 year old female who was originally diagnosed by the University of Pittsburgh Medical School Alzheimer's Disease Research Center to be suffering from a fulminating form of Alzheimer's Disease. In one year, the subject had gone from being director of nursing in a chronic care establishment to requiring constant care. The subject was unable to communicate, did not appear to recognize anyone and had a score of 1.5 on the Mini-Mental State Exam. The subject was treated by sublingual administration four times daily of the amyloid beta dosage unit of Example 1. After three months of treatment, the patient's husband reported that warmth had returned to the patient's hands, no deterioration of any type was evident, although prior to therapy, he could note weekly declines. Communication remained difficult but improved for the subject, she showed increased alertness, and she was not only able to recognize individuals consistently, but also was able, at times, to participate in conversations, and her test score rose to 7.75 on the Mini-Mental State Exam. Although the subject was originally diagnosed as having Alzheimer's Disease, the results of an autopsy indicated that she did not have Alzheimer's Disease but suffered from a form of Parkinson's Disease known as Striatonigral degeneration.

**EXAMPLE 4**

In this example, the subject was a 79 year old male who had suffered from two transient ischemic attacks and had also been diagnosed as having Alzheimer's Disease. Prior to treatment, the subject had a score of 16 on the Mini-Mental State Exam. The subject was treated by sublingual administration four times daily of the amyloid beta dosage unit of Example 1. While the subject became somewhat more irascible and eventually died of a stroke two months after the trial period, his performance on various mental performance exams including the Dementia Rating Scale improved during the initial six month evaluation period. Specifically, the Mini-Mental State Exam improved during the initial six months of testing with scores of 18, 20, 21 and 25 at the three, four, five and six month tests, respectively. The subject's mental performance at the three month follow-up examination had declined significantly, with the score on the Mini-Mental State Exam dropping to the level of the original test with a score of 16. The subject's scores on the other mental performance exams also showed marked decline to the original performance levels. The subject died of a stroke prior to the six-month follow-up evaluation.

**EXAMPLE 5**

In this example, a 76 year old male who was diagnosed as having Alzheimer's Disease, was treated by sublingual administration four times daily of the amyloid beta dosage unit of Example 1. The subject's performance on both the Mini-Mental State Exam and the Dementia Rating Scale improved significantly over five months of testing. Test scores on the Mini-Mental State Exam on the first, third, fourth and fifth months were: 20, 20, 20 and 25; while scores on the Dementia Rating Scale were: 115, 117, 122 and 126. The subject showed improvement primarily in areas of attention and conceptualization and in immediate short term memory, with some improvement in verbal fluency. In contrast, his performance did not improve

in the area of delayed memory which remained severely compromised. In addition, there was only slight improvement demonstrated on tasks requiring new learning abilities which was also severely compromised. The subject was unavailable for follow-up study.

5

#### EXAMPLE 6

In this example, the subject was a 74 year old woman who was diagnosed as having Alzheimer's Disease so advanced that she was unable to identify a comb or a key. The subject was disoriented, incontinent and severely hypertensive and required intensive around the clock care from her sister who was a nurse. The subject was treated by sublingual administration four times daily of the amyloid beta dosage unit of Example 1. The subject's initial score on the Mini-Mental State Exam was 2, and the subject demonstrated slight improvements (subsequent scores were 2, 3, 4, 8 and 7) although some, but not all, of the improvement may have resulted from modification of the test procedure to accommodate the marked expressive language deficits exhibited by the subject. Significantly, the subject's blood pressure returned to normal upon treatment with the amyloid protein composition. This indicates that the amyloid protein composition exhibits utility in treatment of the symptoms of atherosclerosis which can be associated with amyloid plaques.

#### B. Application of Materials and Methods to the Treatment of Arteriosclerotic Diseases

Alzheimer's patients treated with amyloid protein as described above show a significant decrease in blood pressure as a result of such treatment which is concomitant with a reduction in symptoms of dementia. As noted above, Melnick, *et al.* report a role for a herpesvirus (Cytomegalovirus, CMV) in atherogenesis. In co-owned, United States Patent No. 4,880,626, it is noted that, while all untreated AIDS patients studied had CMV, none of the patients treated with a influenza vaccine based (Fluogen™)

25  
30



composition had CMV. The present application teaches compositions and methods comprising the anti-plaquing amyloid protein and the anti-viral thimerosal in order to effect treatment of patients presenting with arteriosclerotic conditions. The following examples provide exemplification of the invention through representative embodiments comprising the use of claimed compositions and methods on human subjects. For each example below, original (*i.e.*, pretreatment) blood pressure was taken about three times during each reading to ensure accuracy. Subsequent measures were repeated about 5 times. Unless otherwise noted, patients undergoing treatment according to the invention received 1 drop (sublingual) four times per day. One drop is approximately 0.05 mL of a composition according to the invention.

#### EXAMPLE 7

A 54-year-old male patient presented with atherosclerosis, including blood pressure of 140/90. The patient was treated with sublingual drops of a composition comprising  $10^{-9}$  mg amyloid protein in a 1:25 dilution of 0.05 mL thimerosal containing influenza vaccine (Fluogen™) in saline. The patient was not treated with any other medication during the period in which he was treated with the above composition. In addition, the patient reported that he remained on a high fat diet and reported no exercise during the treatment period. After daily sublingual treatment (1 drop 4 times per day) for 90 days, the patient's blood pressure had decreased to 117/72. After two years of taking the above composition, the patient's blood pressure has stabilized at about 115/70. The patient's cholesterol also significantly decreased after sustained treatment.

#### EXAMPLE 8

A 44-year-old, moderately obese male presented with blood pressure of 140/110. The patient was treated with daily sublingual doses (4

times daily) of a composition according to the invention, as recited above in Example 1. The patient received no other medication and did not otherwise alter his lifestyle during the treatment period with the result that his blood pressure had decreased to 120/90. After continued treatment as described  
5 above and with no change in lifestyle or diet during the treatment period, the patient had a blood pressure of 123/78. Blood pressure was taken up to five times during each measurement to ensure accuracy.

#### EXAMPLE 9

10 A 55-year old female with initial (pretreatment) blood pressure of about 138/90 began treatment according to methods of the invention and with compositions according to the invention. The patient showed a steady improvement in the diastolic component of blood pressure through three months of treatment. At that point, the patient discontinued treatment and  
15 three months later showed increases in diastolic blood pressure. Upon resuming treatment, diastolic blood pressure again decreased. During treatment, the patient was administered compositions as described above in Examples 1 and 2. The following table provides a partial tracking of the patient's blood pressure during treatment and non-treatment periods.

20

25

30

**TABLE 1**

	Treatment	Blood Pressure
	pre-treatment	138/90
5	yes	150/88
	yes	144/82
	no	136/82
	no	118/74
	no	122/80
10	no	142/86
	yes	140/80
	yes	130/78

15

**EXAMPLE 10**

A 50-year-old male patient presented with blood pressure of 150/104 and began treatment as described above. The results of that treatment are presented in Table 2.

20

25

30

TABLE 2

	Treatment	Blood Pressure
	yes	146/95
5	yes	155/94
	yes	138/90
	yes	138/78
	yes*	150/98
	yes*	140/96
10	yes*	156/108
	no	160/102
	no	160/100
	yes	130/78

\*dose of 1-2 drops/day

15

The results presented in Examples 3 and 4 demonstrate not only the effect of compositions according to the invention in reducing symptoms of arteriosclerosis, but also demonstrate that such symptoms return upon cessation of treatment according to the invention.

20

#### EXAMPLE 11

A 76-year-old female with blood pressure of 210/110 began treatment according to the invention and as described above, using 4 drops per day. After one month of treatment, the patient's blood pressure was reduced to 200/90. After 90 days of treatment her blood pressure was reduced to 160/90.

25

30

**EXAMPLE 12**

According to this example veterinary clinical trials were carried out involving the administration of amyloid beta protein and a thimerosal containing fraction of influenza virus vaccine for the treatment of atherosclerosis in a rabbit atherosclerosis model. Specifically, a filter centrifugation technique was used to isolate a 30kD fraction of commercially available influenza virus vaccine (Fluviron™) containing thimerosal as a preservative at a concentration of 0.01 % wherein the vaccine was loaded onto an Ultrafree low-binding spin-filter unit with a 30,000 nominal molecular weight limit and centrifuged in a microfuge until all of the fluid had passed through the filter. The filtrate was further filtered through a 5,000 nominal molecular weight spin filter to yield a thimerosal containing 5kD filtrate fraction.

According to the trial, four groups of six atherosclerosis-prone Watanabe rabbits apiece were treated with A) saline as a control; B) a composition according to the invention comprising  $10^{-4}$  mg of amyloid beta protein and the 5kD thimerosal containing influenza virus vaccine fraction; C) a composition according to the invention comprising  $10^{-3}$  mg amyloid beta protein and the 5kD thimerosal containing influenza virus vaccine fraction; and D) a composition according to the invention comprising  $10^{-4}$  mg of amyloid beta protein and the 5kD thimerosal containing influenza virus vaccine fraction. The animals were treated for six months at which time the surviving animals were tested for weight gain and two animals for each group were sacrificed and vessel patency was determined by a gross pathological examination of plaque formation in the lumen of the aorta. The animals treated with the compositions of the invention had clear bifurcations and exhibited fewer and smaller plaques than did the control animals which were aged and sex matched and which exhibited plaque accumulation and occlusion of vessels exiting the aorta. The average weight gain over six months for the control animals was 690 grams as compared to the three test groups which

showed, respectively, average weight gains of 450 grams (Group B); 450 grams (Group C); and 525 grams (Group D).

### EXAMPLE 13

5                   According to this example, a U.S. FDA Phase 1/2 controlled double blind human clinical trial was conducted using thimerosal containing compositions for the treatment of chronic fatigue syndrome. Thirty-six (36) patients suffering from documented chronic fatigue syndrome were studied of whom thirty-three (33) completed the study. Of the subjects who completed  
10 the study, 17 were treated with placebo and 16 were treated by sublingual administration six times daily of 1 drop (0.05 mL) of a composition comprising 0.0020 mL (2  $\mu$ L) influenza vaccine containing 0.01 % (0.2  $\mu$ g) thimerosal, 0.0004 mL (0.4  $\mu$ L) rubella virus vaccine and 0.0576 mL saline. After ten weeks of treatment, the subjects were evaluated and were taken off  
15 of either the drug or placebo and were further evaluated after an additional four weeks of no treatment.

                  The subjects were evaluated by means of two principal art recognized efficacy parameters: (1) a visual analogue scale for subjective evaluation of fatigue, and (2) a fatigue impact scale comprising 36 questions  
20 related to cognitive, psychologic and social disorders. Analysis of the results using the visual analogue scale showed no statistically significant difference at the 95 % confidence level between the therapy and the control. Analysis of the results using the fatigue impact scale also failed to demonstrate a statistically significant difference at the 95 % confidence level between the  
25 therapy and placebo groups but did indicate a trend in favor of the therapy over the placebo indicative of a therapeutic effect.

C. Application of Materials and Methods of the Invention to the Treatment of Herpes Infections

The following examples relate to work establishing the *in vitro* and *in vivo* anti-herpes viral activity of thimerosal as the anti-herpes active fraction of influenza virus vaccines.

Typically, a pharmaceutical dosage unit of the present invention for the delivery of thimerosal comprises a liquid or solid carrier and an effective amount of thimerosal. One suitable carrier for sublingual administration comprises a phenylated saline solution. Effective amounts of thimerosal range from about 0.05  $\mu\text{g}$  to 500  $\mu\text{g}$  thimerosal with about 0.5  $\mu\text{g}$  to about 50  $\mu\text{g}$  thimerosal being preferred and about 5  $\mu\text{g}$  thimerosal being particularly preferred. The thimerosal is preferably administered in association with pharmaceutically acceptable excipients. The thimerosal is administered through standard methods, including sublingual, subcutaneous and transdermal routes, and in dosage units that are either liquid or solid.

**EXAMPLE 14**

According to this example, a filter centrifugation technique was used to isolate a 30kD fraction of commercially available influenza virus vaccines (Fluviron™ and Fluzone™) wherein the vaccine was loaded onto an Ultrafree low-binding spin-filter unit with a 30,000 nominal molecular weight limit and centrifuged in a microfuge until all of the fluid had passed through the filter. *In vitro* assays with the 30kD filtrate fractions (which contained thimerosal present at a concentration of 0.01% as a preservative in the commercial vaccine) saw complete inhibition of herpes virus in cell culture assay utilizing HSV-1 and HSV-2 infection of A549 (Human Lung Carcinoma) cells *in vitro*. The fraction obtained was also used in place of dilute influenza virus vaccine in human subjects and was found to improve the clinical response to chronic fatigue syndrome and other herpes infections.

**EXAMPLE 15**

According to this example, a further filtration was carried out isolating a 5kD fraction from the Fluvirin™ influenza vaccine. This fraction which also contained thimerosal was effective at inhibiting growth of the herpes virus in the cell culture of Example 14 and appeared to clinically superior in *in vivo* administration to 30kD fraction of Example 14.

In the following set of examples 16 through 21 the 30kD thimerosal containing influenza vaccine isolate was administered to subjects suffering from herpes virus infections.

10

**EXAMPLE 16**

In this example, a 38 year old female presented with acute onset of pain on the left side of the head, neck, shoulder, chest and arm which she had suffered for five days. She also suffered from similar pain on the dorsal left foot. Examination revealed three small papular lesions in a quarter-sized red area on the left upper chest. Herpes zoster was suspected. The 30kD thimerosal containing fraction of example 15 was administered sublingually with one drop (0.05 mL) four times daily for a dosage of about 5 µg thimerosal. The subject reported a 95% reduction in pain 30 to 60 minutes after administration of the thimerosal containing fraction. The subject continued to do well after one week of QID treatment with one drop of the composition.

15

20

**EXAMPLE 17**

In this example a 66 year old female with a history of recurrent herpes zoster presented during the initial stages of such an outbreak. Administration of one drop sublingually of the composition of Example 16 eliminated discomfort. The subject took a second drop of the composition 24 hours later and did not experience further pain or discomfort for the next two weeks.

25

30



**EXAMPLE 18**

In this example a 19 year old male presented with a history of having developed acute infectious mononucleosis approximately five days previously with symptoms of sore throat, 4+ fatigue, lymph node enlargement (4+ cervical), headache, dizziness, splenomegaly. The subject was treated with one subcutaneous injection of 0.2 mL of the composition of example 16 and reported 60-70% improvement within 30 minutes. Seven hours later, the subject reported feeling 90% better.

**EXAMPLE 19**

According to this example a 30 year old woman presented with predictable oral herpes simplex outbreak on the 23rd day of her menstrual cycle. Associated with the herpes outbreak were premenstrual syndrome symptoms which were sufficiently severe as to require anti-psychotic medication. The patient administered 2 drops of the composition of Example 16 sublingually at the first sign of herpes outbreak (that being sensitivity of the lip in the area of the typical lesion expression). No cold sores developed in or on her mouth for the first time in several years and the premenstrual syndrome was not exhibited.

**EXAMPLE 20**

A 63 year old female subject with a history of lesions lasting several weeks presented with a four day old HSV-1 lesion on her lip. One drop of the composition of example 16 was administered sublingually and within 30 minutes the subject reported a marked improvement. The lesion resolved in two days with administration of two drops of the composition per day.

**EXAMPLE 21**

A 50 year old plus male presented with chronic fatigue syndrome having pronounced lethargy, a history of mental foggiess and poor quality of life. Two drops of the composition of example 16 were administered to the subject sublingually and the subject reported improvement in excess of 70% with an increase in energy and mental clarity for the first time in several years. After three weeks, the subject continued to do well with administration of two drops of the composition daily.

**EXAMPLE 22**

According to this example, a wide variety of tests were carried out to determine the anti-herpes active component of the 5kD influenza vaccine fraction with the result that it was determined that thimerosal possesses the anti-herpes effect. *In vitro* assays with purified thimerosal demonstrate activity against herpes viruses. Of interest is the observation that when thimerosal is mixed in a test tube with herpes virus and is subsequently introduced into susceptible cells viral propagation is not inhibited. This suggests that the anti-herpes viral activity of thimerosal is not by direct action on the virus. Further study shows that thiosalicylic acid and dithiodibenzoic acid which are the breakdown products of thimerosal do not provide antiviral activity *in vivo* although dithiodibenzoic acid shows anti-herpes virus activity *in vitro*.

**EXAMPLE 23**

According to this example, a double-blind study was carried out on sixteen subjects suffering with chronic fatigue syndrome with three compositions designated A, B, and C administered over the course of one month as daily sublingual drops. Each subject was treated by sublingual administration of one drop (0.05 ml) four to six times daily. Composition A comprised 0.0004% weight per volume thimerosal (0.2  $\mu$ g per drop) and 3.2

x  $10^{-7}$  units neuraminidase per drop in saline. Composition B comprised 0.0004% weight per volume thimerosal (0.2  $\mu\text{g}$  per drop) in combination with 0.00032 mL (0.32  $\mu\text{L}$ ) rubella virus vaccine (Meruvax, Merck & Co.) in saline. Composition C comprised 0.0004% weight per volume thimerosal (0.2  $\mu\text{g}$  per drop) alone in saline. The following parameters were measured by patients' self-reported scores: overall level of fatigue; overall level of pain; severity of flare-ups; muscle cramps; headaches; mental alertness and memory; and overall or average sleep. According to evaluation of these parameters, compositions A and B exhibited significant improvements in the severity of CFS symptoms. Moreover, if the results of one subject treated with composition B are omitted (because physical therapy starting and ending about the same time as the therapy may have adversely affected the results for that subject) the remaining subjects treated with composition B comprising the combination of thimerosal and rubella virus vaccine exhibited the greatest decrease in the severity of chronic fatigue syndrome symptoms.

The foregoing representative results demonstrate that application of compositions according to the invention reduce blood pressure and other symptoms associated with arteriosclerosis. Therefore, treatment methods and compositions according to the invention constitute an effective means for alleviating the symptoms of arteriosclerotic diseases and for completely alleviating such diseases in some cases.

## WHAT IS CLAIMED IS:

1. A method for alleviating the symptoms of disease states associated with amyloid plaque formation and/or formation of arterial plaques comprising the step of administering to a patient of an effective amount of amyloid protein or a therapeutically-active fragment thereof and thimerosal free of association with influenza virus.  
5
2. The method of claim 1 wherein the amyloid protein is an amyloid beta protein.  
10
3. The method of claim 1 wherein the amyloid protein is a fragment comprising the first 28 amino acid residues of the amyloid beta protein.  
15
4. The method of claim 1 wherein from about  $10^{-10}$  to about  $10^{-2}$  mg of amyloid protein is administered per dose.
5. The method of claim 1 wherein from about  $10^{-5}$  to about  $10^{-3}$  mg of amyloid protein is administered per dose.  
20
6. The method of claim 1 wherein the disease state is associated with abnormal accumulation of and/or molecular organization of amyloid protein or amyloid plaques associated with central nervous system and histopathologically related disorders.  
25
7. The method of claim 1 wherein the disease state is Alzheimer's Disease.

8. The method of claim 1 wherein the disease state is Parkinson's Disease.

5 9. The method of claim 1 wherein the disease state is atherosclerosis.

10 10. The method of claim 1 wherein said composition comprises from about  $10^{-10}$  to about  $10^{-2}$  mg of amyloid protein and from about 0.05  $\mu$ g to about 500  $\mu$ g thimerosal.

11. The method of claim 10 wherein said composition is administered to a patient in an amount of about 0.05 cc of the composition.

15 12. A pharmaceutical composition for treatment of disease states associated with abnormal accumulation of and/or molecular organization of amyloid protein or amyloid plaques which comprises amyloid protein or a therapeutically active fragment thereof, thimerosal free of association with influenza virus in amounts effective to alleviate one or more symptoms of said disease state and a suitable carrier.

20 13. The pharmaceutical composition of claim 12 wherein the amyloid protein is amyloid beta protein.

25 14. The pharmaceutical composition of claim 12 wherein the amyloid protein is a fragment comprising the first 28 amino acid residues of the amyloid beta protein.

30 15. The pharmaceutical composition of claim 12 in the form of a single dosage unit which comprises from about  $10^{-10}$  to about  $10^{-2}$  mg of amyloid protein and from about 0.05  $\mu$ g to about 500  $\mu$ g of thimerosal.

16. A method for treating hypertension, comprising administration of an effective amount of a pharmaceutical composition comprising amyloid protein or a therapeutically active fragment thereof and from about 0.05  $\mu\text{g}$  to about 500  $\mu\text{g}$  of thimerosal free of association with influenza virus.

17. A method for treating subjects suffering from herpes virus infections, comprising the step of administering an effective amount of a composition comprising thimerosal free of association with influenza virus.

18. The method of claim 17 wherein the subjects is suffering from chronic fatigue syndrome.

19. The method of claim 17 wherein from 0.05  $\mu\text{g}$  to 500  $\mu\text{g}$  of thimerosal is administered per dose.

20. The method of claim 17 wherein from 0.5  $\mu\text{g}$  to 50  $\mu\text{g}$  of thimerosal is administered per dose.

21. The method of claim 18 wherein the composition further comprises a rubella virus vaccine.

22. The method of claim 21 wherein the composition comprises from about 0.01 to about 10 TCID<sub>50</sub> of a rubella virus.

23. The method of claim 17 wherein said composition is administered to a patient in a single dose of 0.05 cc in a pharmaceutically acceptable carrier.

35

24. The method of claim 17 wherein the composition is administered sublingually.

25. A method for *in vitro* killing of herpes virus comprising  
5 administering an effective amount of thimerosal.

10

15

20

25

30

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/14005

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet.

US CL : 514/12, 21; 424/, 94.64, 206.1; 530/324

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 21; 424/, 94.64, 206.1; 530/324

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
none

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4,666,829 A (GLENNER et al.) 19 May 1987, see entire document.	13-16
X	US 5,187,153 A (CORDELL et al.) 16 February 1993, see entire document.	1-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

22 SEPTEMBER 1997

Date of mailing of the international search report

28 OCT 1997

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

P. LYNN TOUZEAU, Ph.D.

Telephone No. (703) 308-0196



**INTERNATIONAL SEARCH REPORT**

International application No.  
PCT/US97/14005

**A. CLASSIFICATION OF SUBJECT MATTER:**  
**IPC (6):**

**A61K 38/00, 38/55; C07K 14/00**



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C07K 14/47, A61K 38/17, G01N 33/68</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/08868</b> <b>(43) International Publication Date:</b> 5 March 1998 (05.03.98)
<b>(21) International Application Number:</b> PCT/US97/15166 <b>(22) International Filing Date:</b> 27 August 1997 (27.08.97)  <b>(30) Priority Data:</b> 08/703,675           27 August 1996 (27.08.96)      US 08/897,342           21 July 1997 (21.07.97)         US  <b>(71) Applicant:</b> PRAECIS PHARMACEUTICALS INCORPORATED [US/US]; One Hampshire Street, Cambridge, MA 02139 (US).	<b>(72) Inventors:</b> FINDEIS, Mark, A.; Apartment 3A, 45 Trowbridge Street, Cambridge, MA 02138 (US). GEFTER, Malcolm, L.; 46 Baker Bridge Road, Lincoln, MA 01773 (US). MUSSO, Gary; 38 Proctor Street, Hopkinton, MA 01748 (US). SIGNER, Ethan, R.; 20 Forest Street, Cambridge, MA 02140 (US). WAKEFIELD, James; 1862 Beacon Street, 1-B2, Brookline, MA 02146 (US). MOLINEAUX, Susan; 69 Centre Street, Brookline, MA 02146 (US). CHIN, Joseph; 190 Loring Avenue, Salem, MA 01970 (US). LEE, Jung-Ja; 261 Cochituate Road, Wayland, MA 01778 (US). KELLEY, Michael; 15 Florence Avenue, Arlington, MA 02174 (US). KOMAR-PANICUCCI, Sonja; 327 Old Billerica Road, Bedford, MA 01730 (US). ARICO-MUENDEL, Christopher, C.; 42 Hillcrest Circle, Watertown, MA 02139 (US). PHILLIPS, Kathryn; Apartment 10, 39 Royal Crest Drive, Marlborough, MA 01752 (US). HAYWARD, Neil, J.; 17 Stoneybrook Road, North Grafton, MA 01536 (US).  <b>(74) Agents:</b> KARA, Catherine, J. et al.; Lahive & Cockfield, LLP, 28 State Street, Boston, MA 02109 (US).  <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(54) Title:</b> MODULATORS OF $\beta$ -AMYLOID PEPTIDE AGGREGATION COMPRISING D-AMINO ACIDS  <b>(57) Abstract</b> <p>Compounds that modulate natural <math>\beta</math>-amyloid peptide aggregation are provided. The modulators of the invention comprise a peptide, preferably based on a <math>\beta</math>-amyloid peptide, that is comprised entirely of D-amino acids. Preferably, the peptide comprises 3-5 D-amino acid residues and includes at least two D-amino acid residues independently selected from the group consisting of D-leucine, D-phenylalanine and D-valine. In a particularly preferred embodiment, the peptide is a retro-inverso isomer of a <math>\beta</math>-amyloid peptide, preferably a retro-inverso isomer of A<math>\beta</math><sub>17-21</sub>. In certain embodiments, the peptide is modified at the amino-terminus, the carboxy-terminus, or both. Preferred amino-terminal modifying groups include cyclic, heterocyclic, polycyclic and branched alkyl groups. Preferred carboxy-terminal modifying groups include an amide group, an alkyl amide group, an aryl amide group or a hydroxy group. Pharmaceutical compositions comprising the compounds of the invention, and diagnostic and treatment methods for amyloidogenic diseases using the compounds of the invention, are also disclosed.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

- 1 -

## MODULATORS OF $\beta$ -AMYLOID PEPTIDE AGGREGATION COMPRISING D-AMINO ACIDS

### Background of the Invention

5 Alzheimer's disease (AD), first described by the Bavarian psychiatrist Alois Alzheimer in 1907, is a progressive neurological disorder that begins with short term memory loss and proceeds to disorientation, impairment of judgment and reasoning and, ultimately, dementia. The course of the disease usually leads to death in a severely debilitated, immobile state between four and 12 years after onset. AD has been  
10 estimated to afflict 5 to 11 percent of the population over age 65 and as much as 47 percent of the population over age 85. The societal cost for managing AD is upwards of 80 billion dollars annually, primarily due to the extensive custodial care required for AD patients. Moreover, as adults born during the population boom of the 1940's and 1950's approach the age when AD becomes more prevalent, the control and treatment of AD  
15 will become an even more significant health care problem. Currently, there is no treatment that significantly retards the progression of the disease. For reviews on AD, see Selkoe, D.J. *Sci. Amer.*, November 1991, pp. 68-78; and Yankner, B.A. *et al.* (1991) *N. Eng. J. Med.* 325:1849-1857.

It has recently been reported (Games *et al.* (1995) *Nature* 373:523-527) that an  
20 Alzheimer-type neuropathology has been created in transgenic mice. The transgenic mice express high levels of human mutant amyloid precursor protein and progressively develop many of the pathological conditions associated with AD.

Pathologically, AD is characterized by the presence of distinctive lesions in the victim's brain. These brain lesions include abnormal intracellular filaments called  
25 neurofibrillary tangles (NTFs) and extracellular deposits of amyloidogenic proteins in senile, or amyloid, plaques. Amyloid deposits are also present in the walls of cerebral blood vessels of AD patients. The major protein constituent of amyloid plaques has been identified as a 4 kilodalton peptide called  $\beta$ -amyloid peptide ( $\beta$ -AP)(Glenner, G.G. and Wong, C.W. (1984) *Biochem. Biophys. Res. Commun.* 120:885-890; Masters, C. *et al.* (1985) *Proc. Natl. Acad. Sci. USA* 82:4245-4249). Diffuse deposits of  $\beta$ -AP are  
30 frequently observed in normal adult brains, whereas AD brain tissue is characterized by more compacted, dense-core  $\beta$ -amyloid plaques. (See *e.g.*, Davies, L. *et al.* (1988) *Neurology* 38:1688-1693) These observations suggest that  $\beta$ -AP deposition precedes, and contributes to, the destruction of neurons that occurs in AD. In further support of a  
35 direct pathogenic role for  $\beta$ -AP,  $\beta$ -amyloid has been shown to be toxic to mature

- 2 -

neurons, both in culture and *in vivo*. Yankner, B.A. *et al.* (1989) *Science* 245:417-420; Yankner, B.A. *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:9020-9023; Roher, A.E. *et al.* (1991) *Biochem. Biophys. Res. Commun.* 174:572-579; Kowall, N.W. *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:7247-7251. Furthermore, patients with hereditary cerebral hemorrhage with amyloidosis-Dutch-type (HCHWA-D), which is characterized by diffuse  $\beta$ -amyloid deposits within the cerebral cortex and cerebrovasculature, have been shown to have a point mutation that leads to an amino acid substitution within  $\beta$ -AP. Levy, E. *et al.* (1990) *Science* 248:1124-1126. This observation demonstrates that a specific alteration of the  $\beta$ -AP sequence can cause  $\beta$ -amyloid to be deposited.

10 Natural  $\beta$ -AP is derived by proteolysis from a much larger protein called the amyloid precursor protein (APP). Kang, J. *et al.* (1987) *Nature* 325:733; Goldgaber, D. *et al.* (1987) *Science* 235:877; Robakis, N.K. *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:4190; Tanzi, R.E. *et al.* (1987) *Science* 235:880. The APP gene maps to chromosome 21, thereby providing an explanation for the  $\beta$ -amyloid deposition seen at  
15 an early age in individuals with Down's syndrome, which is caused by trisomy of chromosome 21. Mann, D.M. *et al.* (1989) *Neuropathol. Appl. Neurobiol.* 15:317; Rumble, B. *et al.* (1989) *N. Eng. J. Med.* 320:1446. APP contains a single membrane spanning domain, with a long amino terminal region (about two-thirds of the protein) extending into the extracellular environment and a shorter carboxy-terminal region  
20 projecting into the cytoplasm. Differential splicing of the APP messenger RNA leads to at least five forms of APP, composed of either 563 amino acids (APP-563), 695 amino acids (APP-695), 714 amino acids (APP-714), 751 amino acids (APP-751) or 770 amino acids (APP-770).

Within APP, naturally-occurring  $\beta$  amyloid peptide begins at an aspartic acid  
25 residue at amino acid position 672 of APP-770. Naturally-occurring  $\beta$ -AP derived from proteolysis of APP is 39 to 43 amino acid residues in length, depending on the carboxy-terminal end point, which exhibits heterogeneity. The predominant circulating form of  $\beta$ -AP in the blood and cerebrospinal fluid of both AD patients and normal adults is  $\beta$ 1-40 ("short  $\beta$ "). Seubert, P. *et al.* (1992) *Nature* 359:325; Shoji, M. *et al.* (1992) *Science*  
30 258:126. However,  $\beta$ 1-42 and  $\beta$ 1-43 ("long  $\beta$ ") also are forms in  $\beta$ -amyloid plaques. Masters, C. *et al.* (1985) *Proc. Natl. Acad. Sci. USA* 82:4245; Miller, D. *et al.* (1993) *Arch. Biochem. Biophys.* 301:41; Mori, H. *et al.* (1992) *J. Biol. Chem.* 267:17082. Although the precise molecular mechanism leading to  $\beta$ -APP aggregation and deposition is unknown, the process has been likened to that of nucleation-dependent  
35 polymerizations, such as protein crystallization, microtubule formation and actin

- 3 -

polymerization. See *e.g.*, Jarrett, J.T. and Lansbury, P.T. (1993) *Cell* 73:1055-1058. In such processes, polymerization of monomer components does not occur until nucleus formation. Thus, these processes are characterized by a lag time before aggregation occurs, followed by rapid polymerization after nucleation. Nucleation can be accelerated by the addition of a "seed" or preformed nucleus, which results in rapid polymerization. The long  $\beta$  forms of  $\beta$ -AP have been shown to act as seeds, thereby accelerating polymerization of both long and short  $\beta$ -AP forms. Jarrett, J.T. *et al.* (1993) *Biochemistry* 32:4693.

In one study, in which amino acid substitutions were made in  $\beta$ -AP, two mutant  $\beta$  peptides were reported to interfere with polymerization of non-mutated  $\beta$ -AP when the mutant and non-mutant forms of peptide were mixed. Hilbich, C. *et al.* (1992) *J. Mol. Biol.* 228:460-473. Equimolar amounts of the mutant and non-mutant (*i.e.*, natural)  $\beta$  amyloid peptides were used to see this effect and the mutant peptides were reported to be unsuitable for use *in vivo*. Hilbich, C. *et al.* (1992), *supra*.

### Summary of the Invention

This invention pertains to compounds, and pharmaceutical compositions thereof, that can bind to natural  $\beta$  amyloid peptides ( $\beta$ -AP), modulate the aggregation of natural  $\beta$ -AP and/or inhibit the neurotoxicity of natural  $\beta$ -APs. The  $\beta$ -amyloid modulator compounds of the invention comprise a peptidic structure, preferably based on  $\beta$ -amyloid peptide, that is composed entirely of D-amino acids. In various embodiments, the peptidic structure of the modulator compound comprises a D-amino acid sequence corresponding to a L-amino acid sequence found within natural  $\beta$ -AP, a D-amino acid sequence which is a retro-inverso isomer of an L-amino acid sequence found within natural  $\beta$ -AP or a D-amino acid sequence that is a scrambled or substituted version of an L-amino acid sequence found within natural  $\beta$ -AP. Preferably, the D-amino acid peptidic structure of the modulator is designed based upon a subregion of natural  $\beta$ -AP at positions 17-21 ( $A\beta_{17-20}$  and  $A\beta_{17-21}$ , respectively), which has the amino acid sequences Leu-Val-Phe-Phe-Ala (SEQ ID NO: 3).

A modulator compound of the invention preferably comprises 3-20 D-amino acids, more preferably 3-10 D-amino acids and even more preferably 3-5 D-amino acids. The D-amino acid peptidic structure of the modulator can have free amino- and carboxy-termini. Alternatively, the amino-terminus, the carboxy-terminus or both may be modified. For example, an N-terminal modifying group can be used that enhances the ability of the compound to inhibit  $A\beta$  aggregation. Moreover, the amino- and/or

- 4 -

carboxy termini of the peptide can be modified to alter a pharmacokinetic property of the compound (such as stability, bioavailability and the like). Preferred carboxy-terminal modifying groups include amide groups, alkyl or aryl amide groups (*e.g.*, phenethylamide) and hydroxy groups (*i.e.*, reduction products of peptide acids, resulting in peptide alcohols). Still further, a modulator compound can be modified to label the compound with a detectable substance (*e.g.*, a radioactive label).

In certain preferred embodiments, the invention provides a compound having the structure:

10 A-(Xaa)-B

wherein (Xaa) is a peptidic structure selected from the group consisting of D-Leu-D-Val-D-Phe-D-Phe, D-Leu-D-Val-D-Phe-phenethylamide, D-Leu-D-Val-D-Tyr-D-Phe, D-Leu-D-Val-D-IodoTyr-D-Phe, D-Leu-D-Val-D-Phe-D-Tyr, D-Leu-D-Val-D-Phe-D-IodoTyr, D-Leu-D-Val-D-Phe-D-Ala, D-Leu-D-Val-D-Phe-D-Phe-D-Ala, D-Ala-D-Val-D-Phe-D-Phe-D-Leu, D-Leu-D-Val-D-Tyr-D-Phe-D-Ala, D-Leu-D-Val-D-IodoTyr-D-Phe-D-Ala, D-Leu-D-Val-D-Phe-D-Tyr-D-Ala, D-Leu-D-Val-D-Phe-D-IodoTyr-D-Ala, D-Phe-D-Phe-D-Val-D-Leu, D-Ala-D-Phe-D-Phe-D-Val, D-Ala-D-Phe-D-Phe-D-Val-D-Leu, D-Ala-D-Phe-D-Phe-D-Leu-D-Leu, D-Leu-D-Phe-D-Phe-D-Val-D-Leu, D-Phe-D-Phe-D-Phe-D-Val-D-Leu, D-Phe-D-Phe-D-Phe-D-Leu-D-Val, D-Phe-D-Phe-D-Phe-D-Phe-D-Leu and D-Ala-D-Phe-D-Phe-D-Phe-D-Leu.

A is an amino-terminal modifying group selected from the group consisting of phenylacetyl, diphenylacetyl, triphenylacetyl, butanoyl, isobutanoyl, hexanoyl, propionyl, 3-hydroxybutanoyl, 4-hydroxybutanoyl, 3-hydroxypropionyl, 2, 4-dihydroxybutyryl, 1-Adamantanecarbonyl, 4-methylvaleryl, 2-hydroxyphenylacetyl, 3-hydroxyphenylacetyl, 4-hydroxyphenylacetyl, 3,5-dihydroxy-2-naphthoyl, 3,7-dihydroxy-2-naphthoyl, 2-hydroxycinnamoyl, 3-hydroxycinnamoyl, 4-hydroxycinnamoyl, hydrocinnamoyl, 4-formylcinnamoyl, 3-hydroxy-4-methoxycinnamoyl, 4-hydroxy-3-methoxycinnamoyl, 2-carboxycinnamoyl, 3,4-dihydroxyhydrocinnamoyl, 3,4-dihydroxycinnamoyl, *trans*-Cinnamoyl, (±)-mandelyl, (±)-mandelyl-(±)-mandelyl, glycolyl, 3-formylbenzoyl, 4-formylbenzoyl, 2-formylphenoxyacetyl, 8-formyl-1-naphthoyl, 4-(hydroxymethyl)benzoyl, 3-hydroxybenzoyl, 4-hydroxybenzoyl, 5-hydantoinacetyl, L-hydroorotyl, 2,4-dihydroxybenzoyl, 3-benzoylpropanoyl, (±)-2,4-dihydroxy-3,3-dimethylbutanoyl, DL-3-(4-hydroxyphenyl)lactyl, 3-(2-hydroxyphenyl)propionyl, 4-(2-hydroxyphenyl)propionyl,

- 5 -

D-3-phenyllactyl, 3-(4-hydroxyphenyl)propionyl, L-3-phenyllactyl, 3-pyridylacetyl, 4-pyridylacetyl, isonicotinoyl, 4-quinolinecarboxyl, 1-isoquinolinecarboxyl and 3-isoquinolinecarboxyl.

and B is a carboxy-terminal modifying group selected from the group consisting  
5 of an amide group, an alkyl amide group, an aryl amide group and a hydroxy group.

In another embodiment, (Xaa) is a peptidic structure as described above, B is a  
carboxyl-terminal modifying group as described above and A is an amino-terminal  
modifying group selected from the group consisting of cholesteryl, lithocholesteryl,  
hyodeoxycholesterol, chenodeoxycholesterol and ursodeoxycholesterol. In a preferred  
10 subembodiment, A is selected from the group consisting of lithocholesteryl, hyodeoxycholesterol,  
chenodeoxycholesterol and ursodeoxycholesterol.

Particularly preferred compounds of the invention are set forth in the Examples.

Another aspect of the invention pertains to pharmaceutical compositions.  
Typically, the pharmaceutical composition comprises a therapeutically effective amount  
15 of a modulator compound of the invention and a pharmaceutically acceptable carrier.

Yet another aspect of the invention pertains to methods for inhibiting  
aggregation of natural  $\beta$ -amyloid peptides. These methods comprise contacting the  
natural  $\beta$ -amyloid peptides with a modulator compound of the invention such that  
aggregation of the natural  $\beta$ -amyloid peptides is inhibited.

20 Yet another aspect of the invention pertains to methods for detecting the  
presence or absence of natural  $\beta$ -amyloid peptides in a biological sample. These  
methods comprise contacting a biological sample with a compound of the invention,  
wherein the compound is labeled with a detectable substance, and detecting the  
compound bound to natural  $\beta$ -amyloid peptides to thereby detect the presence or  
25 absence of natural  $\beta$ -amyloid peptides in the biological sample.

Still another aspect of the invention pertains to methods for treating a subject for  
a disorder associated with  $\beta$ -amyloidosis. These methods comprise administering to the  
subject a therapeutically effective amount of a modulator compound of the invention  
such that the subject is treated for a disorder associated with  $\beta$ -amyloidosis. Preferably,  
30 the disorder is Alzheimer's disease. Use of the modulators of the invention for therapy  
or for the manufacture of a medicament for the treatment of a disorder associated with  $\beta$ -  
amyloidosis is also encompassed by the invention.



- 6 -

**Brief Description of the Drawings**

Figure 1 is a bar graph depicting the stability of an L-amino acid-based modulator compound (PPI-368) and two D-amino acid-based modulator compounds (PPI-433 and PPI-457) in cerebrospinal fluid.

5        Figure 2 is a graph depicting the levels of PPI-558 in the plasma at 2, 8 and 24 hours following a single subcutaneous injection of PPI-558 ( 4.6 mg/kg) to male Sprague-Dawley rats. Each point is the mean  $\pm$  standard error for four rats.

Figure 3 is a graph depicting the levels of PPI-558 in the brain parenchyma (void of blood and brain capillaries) at 2, 8 and 24 hours following a single subcutaneous  
10       injection of PPI-558 (4.6 mg/kg) to male Sprague-Dawley rats. Each point is the mean  $\pm$  standard error for four rats.

Figure 4 is a graph depicting the ratio of brain parenchyma versus plasma levels of PPI-558 at 2, 8 and 24 hours following a single subcutaneous injection of PPI-558 (4.6 mg/kg) to male Sprague-Dawley rats. Each point is the mean  $\pm$  standard error for  
15       four rats.

**Detailed Description of the Invention**

This invention pertains to compounds, and pharmaceutical compositions thereof, that can bind to natural  $\beta$ -amyloid peptides, modulate the aggregation of natural  $\beta$   
20       amyloid peptides ( $\beta$ -AP) and/or inhibit the neurotoxicity of natural  $\beta$ -APs. A compound of the invention that modulates aggregation of natural  $\beta$ -AP, referred to herein interchangeably as a  $\beta$  amyloid modulator compound, a  $\beta$  amyloid modulator or simply a modulator, alters the aggregation of natural  $\beta$ -AP when the modulator is contacted with natural  $\beta$ -AP. Thus, a compound of the invention acts to alter the natural  
25       aggregation process or rate for  $\beta$ -AP, thereby disrupting this process. Preferably, the compounds inhibit  $\beta$ -AP aggregation. The compounds of the invention are characterized in that they comprise a peptidic structure composed entirely of D-amino acid residues. This peptidic structure is preferably based on  $\beta$ -amyloid peptide and can comprise, for example, a D-amino acid sequence corresponding to a L-amino acid  
30       sequence found within natural  $\beta$ -AP, a D-amino acid sequence which is a retro-inverso isomer of an L-amino acid sequence found within natural  $\beta$ -AP or a D-amino acid sequence that is a scrambled or substituted version of an L-amino acid sequence found within natural  $\beta$ -AP. The invention encompasses modulator compounds comprising a D-amino acid peptidic structure having free amino- and carboxy-termini, as well as

- 7 -

modulator compounds in which the amino-terminus, the carboxy-terminus, and/or side chain(s) of the peptidic structure are modified.

The  $\beta$  amyloid modulator compounds of the invention can be selected based upon their ability to bind to natural  $\beta$ -amyloid peptides, modulate the aggregation of natural  $\beta$ -AP *in vitro* and/or inhibit the neurotoxicity of natural  $\beta$ -AP fibrils for cultured cells (using assays described herein). Preferred modulator compounds inhibit the aggregation of natural  $\beta$ -AP and/or inhibit the neurotoxicity of natural  $\beta$ -AP. However, modulator compounds selected based on one or both of these properties may have additional properties *in vivo* that may be beneficial in the treatment of amyloidosis. For example, the modulator compound may interfere with processing of natural  $\beta$ -AP (either by direct or indirect protease inhibition) or by modulation of processes that produce toxic  $\beta$ -AP, or other APP fragments, *in vivo*. Alternatively, modulator compounds may be selected based on these latter properties, rather than inhibition of A $\beta$  aggregation *in vitro*. Moreover, modulator compounds of the invention that are selected based upon their interaction with natural  $\beta$ -AP also may interact with APP or with other APP fragments. Still further, a modulator compound of the invention can be characterized by its ability to bind to  $\beta$ -amyloid fibrils (which can be determined, for example, by radiolabeling the compound, contacting the compound with  $\beta$ -amyloid plaque and imaging the compound bound to the plaque), while not significantly altering the aggregation of the  $\beta$ -amyloid fibrils. Such a compound that binds efficiently to  $\beta$ -amyloid fibrils while not significantly altering the aggregation of the  $\beta$ -amyloid fibrils can be used, for example, to detect  $\beta$ -amyloid fibrils (*e.g.*, for diagnostic purposes, as described further herein). It should be appreciated, however, that the ability of a particular compound to bind to  $\beta$ -amyloid fibrils and/or modulate their aggregation may vary depending upon the concentration of the compound. Accordingly, a compound that, at a low concentration, binds to  $\beta$ -amyloid fibrils without altering their aggregation may nevertheless inhibit aggregation of the fibrils at a higher concentration. All such compounds having the property of binding to  $\beta$ -amyloid fibrils and/or modulating the aggregation of the fibrils are intended to be encompassed by the invention.

As used herein, a "modulator" of  $\beta$ -amyloid aggregation is intended to refer to an agent that, when contacted with natural  $\beta$  amyloid peptides, alters the aggregation of the natural  $\beta$  amyloid peptides. The term "aggregation of  $\beta$  amyloid peptides" refers to a process whereby the peptides associate with each other to form a multimeric, largely insoluble complex. The term "aggregation" further is intended to encompass  $\beta$  amyloid fibril formation and also encompasses  $\beta$ -amyloid plaques.

- 8 -

The terms "natural  $\beta$ -amyloid peptide", "natural  $\beta$ -AP" and "natural A $\beta$  peptide", used interchangeably herein, are intended to encompass naturally occurring proteolytic cleavage products of the  $\beta$  amyloid precursor protein (APP) which are involved in  $\beta$ -AP aggregation and  $\beta$ -amyloidosis. These natural peptides include  $\beta$ -amyloid peptides having 39-43 amino acids (*i.e.*, A $\beta$ <sub>1-39</sub>, A $\beta$ <sub>1-40</sub>, A $\beta$ <sub>1-41</sub>, A $\beta$ <sub>1-42</sub> and A $\beta$ <sub>1-43</sub>). The amino-terminal amino acid residue of natural  $\beta$ -AP corresponds to the aspartic acid residue at position 672 of the 770 amino acid residue form of the amyloid precursor protein ("APP-770"). The 43 amino acid long form of natural  $\beta$ -AP has the amino acid sequence

10       DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGGVVIAT  
(also shown in SEQ ID NO: 1), whereas the shorter forms have 1-4 amino acid residues deleted from the carboxy-terminal end. The amino acid sequence of APP-770 from position 672 (*i.e.*, the amino-terminus of natural  $\beta$ -AP) to its C-terminal end (103 amino acids) is shown in SEQ ID NO: 2. The preferred form of natural  $\beta$ -AP for use in the  
15       aggregation assays described herein is A $\beta$ <sub>1-40</sub>.

In the presence of a modulator of the invention, aggregation of natural  $\beta$  amyloid peptides is "altered" or "modulated". The various forms of the term "alteration" or "modulation" are intended to encompass both inhibition of  $\beta$ -AP aggregation and promotion of  $\beta$ -AP aggregation. Aggregation of natural  $\beta$ -AP is "inhibited" in the  
20       presence of the modulator when there is a decrease in the amount and/or rate of  $\beta$ -AP aggregation as compared to the amount and/or rate of  $\beta$ -AP aggregation in the absence of the modulator. The various forms of the term "inhibition" are intended to include both complete and partial inhibition of  $\beta$ -AP aggregation. Inhibition of aggregation can be quantitated as the fold increase in the lag time for aggregation or as the decrease in  
25       the overall plateau level of aggregation (*i.e.*, total amount of aggregation), using an aggregation assay as described in the Examples. In various embodiments, a modulator of the invention increases the lag time of aggregation at least 1.2-fold, 1.5-fold, 1.8-fold, 2-fold, 2.5-fold, 3-fold, 4-fold or 5-fold. In various other embodiments, a modulator of the invention inhibits the plateau level of aggregation at least 10%, 20%, 30%, 40 %, 50  
30       %, 75 % or 100 %.

A modulator which inhibits  $\beta$ -AP aggregation (an "inhibitory modulator compound") can be used to prevent or delay the onset of  $\beta$ -amyloid deposition. Preferably, inhibitory modulator compounds of the invention inhibit the formation and/or activity of neurotoxic aggregates of natural A $\beta$  peptide (*i.e.*, the inhibitory  
35       compounds can be used to inhibit the neurotoxicity of  $\beta$ -AP). Additionally, the

- 9 -

inhibitory compounds of the invention preferably reduce the neurotoxicity of preformed  $\beta$ -AP aggregates, indicating that the inhibitory modulators can either bind to preformed  $\beta$ -AP fibrils or soluble aggregate and modulate their inherent neurotoxicity or that the modulators can perturb the equilibrium between monomeric and aggregated forms of  $\beta$ -AP in favor of the non-neurotoxic form.

Alternatively, in another embodiment, a modulator compound of the invention promotes the aggregation of natural  $A\beta$  peptides. The various forms of the term "promotion" refer to an increase in the amount and/or rate of  $\beta$ -AP aggregation in the presence of the modulator, as compared to the amount and/or rate of  $\beta$ -AP aggregation in the absence of the modulator. Such a compound which promotes  $A\beta$  aggregation is referred to as a stimulatory modulator compound. Stimulatory modulator compounds may be useful for sequestering  $\beta$ -amyloid peptides, for example in a biological compartment where aggregation of  $\beta$ -AP may not be deleterious to thereby deplete  $\beta$ -AP from a biological compartment where aggregation of  $\beta$ -AP is deleterious. Moreover, stimulatory modulator compounds can be used to promote  $A\beta$  aggregation in *in vitro* aggregation assays (*e.g.*, assays such as those described in Example 2), for example in screening assays for test compounds that can then inhibit or reverse this  $A\beta$  aggregation (*i.e.*, a stimulatory modulator compound can act as a "seed" to promote the formation of  $A\beta$  aggregates).

In a preferred embodiment, the modulators of the invention are capable of altering  $\beta$ -AP aggregation when contacted with a molar excess amount of natural  $\beta$ -AP. A "molar excess amount of natural  $\beta$ -AP" refers to a concentration of natural  $\beta$ -AP, in moles, that is greater than the concentration, in moles, of the modulator. For example, if the modulator and  $\beta$ -AP are both present at a concentration of 1  $\mu$ M, they are said to be "equimolar", whereas if the modulator is present at a concentration of 1  $\mu$ M and the  $\beta$ -AP is present at a concentration of 5  $\mu$ M, the  $\beta$ -AP is said to be present at a 5-fold molar excess amount compared to the modulator. In preferred embodiments, a modulator of the invention is effective at altering natural  $\beta$ -AP aggregation when the natural  $\beta$ -AP is present at at least a 2-fold, 3-fold or 5-fold molar excess compared to the concentration of the modulator. In other embodiments, the modulator is effective at altering  $\beta$ -AP aggregation when the natural  $\beta$ -AP is present at at least a 10-fold, 20-fold, 33-fold, 50-fold, 100-fold, 500-fold or 1000-fold molar excess compared to the concentration of the modulator.

As used herein, the term " $\beta$  amyloid peptide comprised entirely of D-amino acids", as used in a modulator of the invention, is intended to encompass peptides

- 10 -

having an amino acid sequence identical to that of the natural sequence in APP, as well as peptides having acceptable amino acid substitutions from the natural sequence, but which is composed of D-amino acids rather than the natural L-amino acids present in natural  $\beta$ -AP. Acceptable amino acid substitutions are those that do not affect the ability of the D-amino acid-containing peptide to alter natural  $\beta$ -AP aggregation. Moreover, particular amino acid substitutions may further contribute to the ability of the peptide to alter natural  $\beta$ -AP aggregation and/or may confer additional beneficial properties on the peptide (*e.g.*, increased solubility, reduced association with other amyloid proteins, etc.). A peptide having an identical amino acid sequence to that found within a parent peptide but in which all L-amino acids have been substituted with all D-amino acids is also referred to as an "inverso" compounds. For example, if a parent peptide is Thr-Ala-Tyr, the inverso form is D-Thr-D-Ala-D-Tyr.

As used herein, the term "retro-inverso isomer of a  $\beta$  amyloid peptide", as used in a modulator of the invention, is intended to encompass peptides in which the sequence of the amino acids is reversed as compared to the sequence in natural  $\beta$ -AP and all L-amino acids are replaced with D-amino acids. For example, if a parent peptide is Thr-Ala-Tyr, the retro-inverso form is D-Tyr-D-Ala-D-Thr. Compared to the parent peptide, a retro-inverso peptide has a reversed backbone while retaining substantially the original spatial conformation of the side chains, resulting in a retro-inverso isomer with a topology that closely resembles the parent peptide. See Goodman *et al.* "Perspectives in Peptide Chemistry" pp. 283-294 (1981). See also U.S. Patent No. 4,522,752 by Sisto for further description of "retro-inverso" peptides.

Various additional aspects of the modulators of the invention, and the uses thereof, are described in further detail in the following subsections.

#### I. Modulator Compounds

In one embodiment, a modulator compound of the invention comprises a  $\beta$ -amyloid peptide, the  $\beta$ -amyloid peptide being comprised entirely of D-amino acids, wherein the compound binds to natural  $\beta$ -amyloid peptides or modulates the aggregation or inhibits the neurotoxicity of natural  $\beta$ -amyloid peptides when contacted with the natural  $\beta$ -amyloid peptides. Preferably, the  $\beta$ -amyloid peptide of the modulator is comprised of 3-20 D-amino acids, more preferably 3-10 D-amino acids, and even more preferably 3-5 D-amino acids. In one embodiment, the  $\beta$ -amyloid peptide of the modulator is amino-terminally modified, for example with a modifying group

- 11 -

comprising a cyclic, heterocyclic, polycyclic or branched alkyl group. Examples of suitable N-terminal modifying groups are described further in subsection II below. In another embodiment, the  $\beta$ -amyloid peptide of the modulator is carboxy-terminally modified, for example the modulator can comprise a peptide amide, a peptide alkyl or aryl amide (*e.g.*, a peptide phenethylamide) or a peptide alcohol. Examples of suitable C-terminal modifying groups are described further in subsections II and III below. The  $\beta$ -amyloid peptide of the modulator may be modified to enhance the ability of the modulator to alter  $\beta$ -AP aggregation or neurotoxicity. Additionally or alternatively,  $\beta$ -amyloid peptide of the modulator may be modified to alter a pharmacokinetic property of the modulator and/or to label the modulator with a detectable substance (described further in subsection III below).

In another embodiment, a modulator compound of the invention comprises a retro-inverso isomer of a  $\beta$ -amyloid peptide, wherein the compound binds to natural  $\beta$ -amyloid peptides or modulates the aggregation or inhibits the neurotoxicity of natural  $\beta$ -amyloid peptides when contacted with the natural  $\beta$ -amyloid peptides. Preferably, the retro-inverso isomer of the  $\beta$ -amyloid peptide is comprised of 3-20 D-amino acids, more preferably 3-10 D-amino acids, and even more preferably 3-5 D-amino acids. In one embodiment, the retro-inverso isomer is amino-terminally modified, for example with a modifying group comprising a cyclic, heterocyclic, polycyclic or branched alkyl group. Examples of suitable N-terminal modifying groups are described further in subsection II below. In another embodiment, the retro-inverso isomer is carboxy-terminally modified, for example with an amide group, an alkyl or aryl amide group (*e.g.*, phenethylamide) or a hydroxy group (*i.e.*, the reduction product of a peptide acid, resulting in a peptide alcohol). Examples of suitable C-terminal modifying groups are described further in subsections II and III below. The retro-inverso isomer may be modified to enhance the ability of the modulator to alter  $\beta$ -AP aggregation or neurotoxicity. Additionally or alternatively, the retro-inverso isomer may be modified to alter a pharmacokinetic property of the modulator and/or to label the modulator with a detectable substance (described further in subsection III below).

The modulators of the invention preferably are designed based upon the amino acid sequence of a subregion of natural  $\beta$ -AP. The term "subregion of a natural  $\beta$ -amyloid peptide" is intended to include amino-terminal and/or carboxy-terminal deletions of natural  $\beta$ -AP. The term "subregion of natural  $\beta$ -AP" is not intended to include full-length natural  $\beta$ -AP (*i.e.*, "subregion" does not include  $A\beta_{1-39}$ ,  $A\beta_{1-40}$ ,  $A\beta_{1-41}$ ,  $A\beta_{1-42}$  and  $A\beta_{1-43}$ ). A preferred subregion of natural  $\beta$ -amyloid peptide is an "A $\beta$

- 12 -

aggregation core domain" (ACD). As used herein, the term "A $\beta$  aggregation core domain" refers to a subregion of a natural  $\beta$ -amyloid peptide that is sufficient to modulate aggregation of natural  $\beta$ -APs when this subregion, in its L-amino acid form, is appropriately modified (*e.g.*, modified at the amino-terminus), as described in detail in U.S. patent application Serial No. 08/548,998 and U.S. patent application Serial No. 08/616,081, the entire contents of each of which are expressly incorporated herein by reference. Preferably, the ACD is modeled after a subregion of natural  $\beta$ -AP that is less than 15 amino acids in length and more preferably is between 3-10 amino acids in length. In various embodiments, the ACD is modeled after a subregion of  $\beta$ -AP that is 10, 9, 8, 7, 6, 5, 4 or 3 amino acids in length. In one embodiment, the subregion of  $\beta$ -AP upon which the ACD is modeled is an internal or carboxy-terminal region of  $\beta$ -AP (*i.e.*, downstream of the amino-terminus at amino acid position 1). In another embodiment, the ACD is modeled after a subregion of  $\beta$ -AP that is hydrophobic. Preferred A $\beta$  aggregation core domains encompass amino acid residues 17-20 or 17-21 of natural  $\beta$ -AP (A $\beta$ <sub>17-20</sub> and A $\beta$ <sub>17-21</sub>, respectively). The amino acid sequences of A $\beta$ <sub>17-20</sub> and A $\beta$ <sub>17-21</sub> are Leu-Val-Phe-Phe (SEQ ID NO: 8) and Leu-Val-Phe-Phe-Ala (SEQ ID NO: 3), respectively.

As demonstrated in the Examples, D-amino acid-containing modulators designed based upon the amino acid sequences of A $\beta$ <sub>17-20</sub> and A $\beta$ <sub>17-21</sub> are particularly effective inhibitors of A $\beta$  aggregation. These modulators can comprises a D-amino acid sequence corresponding to the L-amino acid sequence of A $\beta$ <sub>17-20</sub> or A $\beta$ <sub>17-21</sub>, a D-amino acid sequence which is a retro-inverso isomer of the L-amino acid sequence of A $\beta$ <sub>17-20</sub> or A $\beta$ <sub>17-21</sub>, or a D-amino acid sequence that is a scrambled or substituted version of the L-amino acid sequence of A $\beta$ <sub>17-20</sub> or A $\beta$ <sub>17-21</sub>. The D-amino acid-based modulators may have unmodified amino- and/or carboxy-termini or, alternatively, the amino-terminus, the carboxy-terminus, or both, may be modified (described further below). The peptidic structures of effective modulators generally are hydrophobic and are characterized by the presence of at least two D-amino acid structures independently selected from the group consisting of a D-leucine structure, a D-phenylalanine structure and a D-valine structure. An used herein, the term a "D-amino acid structure" (such as a "D-leucine structure", a "D-phenylalanine structure" or a "D-valine structure") is intended to include the D-amino acid, as well as analogues, derivatives and mimetics of the D-amino acid that maintain the functional activity of the compound (discussed further below). For example, the term "D-phenylalanine structure" is intended to include D-phenylalanine as well as D-pyridylalanine and D-homophenylalanine. The term "D-leucine structure" is

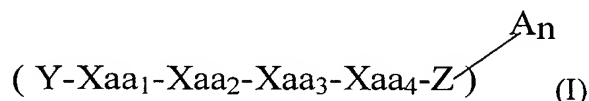
- 13 -

intended to include D-leucine, as well as substitution with D-valine or other natural or non-natural amino acid having an aliphatic side chain, such as D-norleucine. The term "D-valine structure" is intended to include D-valine, as well as substitution with D-leucine or other natural or non-natural amino acid having an aliphatic side chain.

- 5 In other embodiments, the peptidic structure of the modulator comprises at least two D-amino acid structures independently selected from the group consisting of a D-leucine structure, a D-phenylalanine structure, a D-valine structure, a D-alanine structure, a D-tyrosine structure and a D-iodotyrosine structure. In another embodiment, the peptidic structure is comprised of at least three D-amino acid structures
- 10 independently selected from the group consisting of a D-leucine structure, a D-phenylalanine structure and a D-valine structure. In yet another embodiment, the peptidic structure is comprised of at least three D-amino acid structures independently selected from the group consisting of a D-leucine structure, a D-phenylalanine structure, a D-valine structure, a D-alanine structure, a D-tyrosine structure and a D-iodotyrosine
- 15 structure. In yet another embodiment, the peptidic structure comprises at least four D-amino acid structures independently selected from the group consisting of a D-leucine structure, a D-phenylalanine structure and a D-valine structure. In yet another embodiment, the peptidic structure is comprised of at least four D-amino acid structures independently selected from the group consisting of a D-leucine structure, a D-phenylalanine structure and a D-valine structure. In a preferred embodiment, the peptidic structure includes a D-amino acid dipeptide selected from the group consisting of D-Phe-D-Phe, D-Phe-D-Tyr, D-Tyr-D-Phe, D-Phe-D-IodoTyr and D-IodoTyr-D-Phe.
- 20

In one embodiment, the invention provides a  $\beta$ -amyloid modulator compound comprising a formula (I):

25



- wherein Xaa<sub>1</sub>, Xaa<sub>2</sub>, Xaa<sub>3</sub> and Xaa<sub>4</sub> are each D-amino acid structures and at least
- 30 two of Xaa<sub>1</sub>, Xaa<sub>2</sub>, Xaa<sub>3</sub> and Xaa<sub>4</sub> are, independently, selected from the group consisting of a

D-leucine structure, a D-phenylalanine structure and a D-valine structure;

Y, which may or may not be present, is a structure having the formula (Xaa)<sub>a</sub>, wherein Xaa is any D-amino acid structure and a is an integer from 1 to 15;



- 14 -

Z, which may or may not be present, is a structure having the formula (Xaa)<sub>b</sub>, wherein Xaa is any D-amino acid structure and b is an integer from 1 to 15;

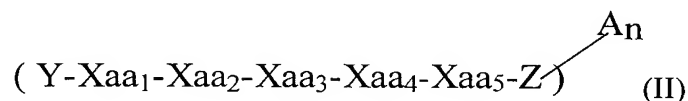
A, which may or may not be present, is a modifying group attached directly or indirectly to the compound; and

5 n is an integer from 1 to 15;

wherein Xaa<sub>1</sub>, Xaa<sub>2</sub>, Xaa<sub>3</sub>, Xaa<sub>4</sub>, Y, Z, A and n are selected such that the compound binds to natural β-amyloid peptides or modulates the aggregation or inhibits the neurotoxicity of natural β-amyloid peptides when contacted with the natural β-amyloid peptides.

10 In a subembodiment of this formula, a fifth amino acid residue, Xaa<sub>5</sub>, is specified C-terminal to Xaa<sub>4</sub> and Z, which may or may not be present, is a structure having the formula (Xaa)<sub>b</sub>, wherein Xaa is any D-amino acid structure and b is an integer from 1 to 14. Accordingly, the invention provides a β-amyloid modulator compound comprising a formula (II):

15



wherein b is an integer from 1 to 14.

20 In a preferred embodiment, Xaa<sub>1</sub>, Xaa<sub>2</sub>, Xaa<sub>3</sub>, Xaa<sub>4</sub> of formula (I) are selected based on the sequence of Aβ<sub>17-20</sub>, or acceptable substitutions thereof. Accordingly, in preferred embodiments, Xaa<sub>1</sub> is a D-alanine structure or a D-leucine structure, Xaa<sub>2</sub> is a D-valine structure, Xaa<sub>3</sub> is a D-phenylalanine structure, a D-tyrosine structure or a D-iodotyrosine structure and Xaa<sub>4</sub> is a D-phenylalanine structure, a D-tyrosine structure or a D-iodotyrosine structure.

25 In another preferred embodiment, Xaa<sub>1</sub>, Xaa<sub>2</sub>, Xaa<sub>3</sub>, Xaa<sub>4</sub> and Xaa<sub>5</sub> of formula (II) are selected based on the sequence of Aβ<sub>17-21</sub>, or acceptable substitutions thereof. Accordingly, in preferred embodiments, Xaa<sub>1</sub> is a D-alanine structure or a D-leucine structure, Xaa<sub>2</sub> is a D-valine structure, Xaa<sub>3</sub> is a D-phenylalanine structure, a D-tyrosine structure or a D-iodotyrosine structure, Xaa<sub>4</sub> is a D-phenylalanine structure, a D-tyrosine structure or a D-iodotyrosine structure, and Xaa<sub>5</sub> is a D-alanine structure or a D-leucine structure.

30

In another preferred embodiment, Xaa<sub>1</sub>, Xaa<sub>2</sub>, Xaa<sub>3</sub> and Xaa<sub>4</sub> of formula (I) are selected based on the retro-inverso isomer of Aβ<sub>17-20</sub>, or acceptable substitutions thereof. Accordingly, in preferred embodiments, Xaa<sub>1</sub> is a D-alanine structure, a D-

- 15 -

leucine structure or a D-phenylalanine structure, Xaa<sub>2</sub> is a D-phenylalanine structure, a D-tyrosine structure or a D-iodotyrosine structure, Xaa<sub>3</sub> is a D-phenylalanine structure, a D-tyrosine structure or a D-iodotyrosine structure and Xaa<sub>4</sub> is a D-valine structure or a D-leucine structure.

5 In another preferred embodiment, Xaa<sub>1</sub>, Xaa<sub>2</sub>, Xaa<sub>3</sub>, Xaa<sub>4</sub> and Xaa<sub>5</sub> of formula (II) are selected based on the retroinverso isomer of Aβ<sub>17-21</sub>, or acceptable substitutions thereof. Accordingly, in preferred embodiments, Xaa<sub>1</sub> is a D-alanine structure, a D-leucine structure or a D-phenylalanine structure, Xaa<sub>2</sub> is a D-phenylalanine structure, a D-tyrosine structure or a D-iodotyrosine structure, Xaa<sub>3</sub> is a D-phenylalanine structure, a  
10 D-tyrosine structure or a D-iodotyrosine structure, Xaa<sub>4</sub> is a D-valine structure or a D-leucine structure and Xaa<sub>5</sub> is a D-leucine structure.

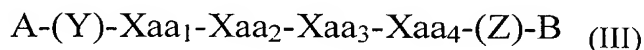
In the modulators of the invention having the formula (I) or (II) shown above, an optional modifying group ("A") is attached directly or indirectly to the peptidic structure of the modulator. (As used herein, the term "modulating group" and "modifying group"  
15 are used interchangeably to describe a chemical group directly or indirectly attached to a peptidic structure). For example, a modifying group(s) can be directly attached by covalent coupling to the peptidic structure or a modifying group(s) can be attached indirectly by a stable non-covalent association. In one embodiment of the invention, a modifying group is attached to the amino-terminus of the peptidic structure of the  
20 modulator. Alternatively, in another embodiment of the invention, a modifying group is attached to the carboxy-terminus of the peptidic structure of the modulator. In yet another embodiment, a modulating group(s) is attached to the side chain of at least one amino acid residues of the peptidic structure of the modulator (*e.g.*, through the epsilon amino group of a lysyl residue(s), through the carboxyl group of an aspartic acid  
25 residue(s) or a glutamic acid residue(s), through a hydroxy group of a tyrosyl residue(s), a serine residue(s) or a threonine residue(s) or other suitable reactive group on an amino acid side chain).

If a modifying group(s) is present, the modifying group is selected such that the compound inhibits aggregation of natural β-amyloid peptides when contacted with the  
30 natural β-amyloid peptides. Accordingly, since the β-AP peptide of the compound is modified from its natural state, the modifying group "A" as used herein is not intended to include hydrogen. In a modulator of the invention, a single modifying group may be attached to the peptidic structure or multiple modifying groups may be attached to the peptidic structure. The number of modifying groups is selected such that the compound  
35 inhibits aggregation of natural β-amyloid peptides when contacted with the natural β-

- 16 -

amyloid peptides. However, n preferably is an integer between 1 and 60, more preferably between 1 and 30 and even more preferably between 1 and 10 or 1 and 5. In a preferred embodiment, A is an amino-terminal modifying group comprising a cyclic, heterocyclic, polycyclic or branched alkyl group and n=1. In another preferred embodiment, A is carboxy-terminally modifying group comprising an amide group, an alkyl amide group, an aryl amide group or a hydroxy group, and n=1. Suitable modifying groups are described further in subsections II and III below.

In another embodiment, the invention provides a  $\beta$ -amyloid modulator compound comprising a formula (III):



wherein  $Xaa_1$ ,  $Xaa_2$ ,  $Xaa_3$  and  $Xaa_4$  are each D-amino acid structures and at least two of  $Xaa_1$ ,  $Xaa_2$ ,  $Xaa_3$  and  $Xaa_4$  are, independently, selected from the group

consisting of a

D-leucine structure, a D-phenylalanine structure and a D-valine structure;

Y, which may or may not be present, is a peptidic structure having the formula  $(Xaa)_a$ , wherein Xaa is any amino acid structure and a is an integer from 1 to 15;

Z, which may or may not be present, is a peptidic structure having the formula  $(Xaa)_b$ , wherein Xaa is any amino acid structure and b is an integer from 1 to 15; and

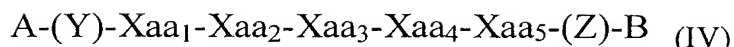
A, which may or may not be present, is a modifying group attached directly or indirectly to the amino terminus of the compound; and

B, which may or may not be present, is a modifying group attached directly or indirectly to the carboxy terminus of the compound;

$Xaa_1$ ,  $Xaa_2$ ,  $Xaa_3$ ,  $Xaa_4$ , Y, Z, A and B being selected such that the compound binds to natural  $\beta$ -amyloid peptides or modulates the aggregation or inhibits the neurotoxicity of natural  $\beta$ -amyloid peptides when contacted with the natural  $\beta$ -amyloid peptides.

In a subembodiment of formula (III), a fifth amino acid residue,  $Xaa_5$ , is specified C-terminal to  $Xaa_4$  and Z, which may or may not be present, is a structure having the formula  $(Xaa)_b$ , wherein Xaa is any D-amino acid structure and b is an integer from 1 to 14. Accordingly, the invention provides a  $\beta$ -amyloid modulator compound comprising a formula (IV):

- 17 -



wherein b is an integer from 1 to 14

5 In a preferred embodiment, Xaa<sub>1</sub>, Xaa<sub>2</sub>, Xaa<sub>3</sub>, Xaa<sub>4</sub> of formula (III) are selected based on the sequence of Aβ<sub>17-20</sub>, or acceptable substitutions thereof. Accordingly, in preferred embodiments, Xaa<sub>1</sub> is a D-alanine structure or a D-leucine structure, Xaa<sub>2</sub> is a D-valine structure, Xaa<sub>3</sub> is a D-phenylalanine structure, a D-tyrosine structure or a D-iodotyrosine structure and Xaa<sub>4</sub> is a D-phenylalanine structure, a D-tyrosine structure or a D-iodotyrosine structure.

10 In another preferred embodiment, Xaa<sub>1</sub>, Xaa<sub>2</sub>, Xaa<sub>3</sub>, Xaa<sub>4</sub> and Xaa<sub>5</sub> of formula (IV) are selected based on the sequence of Aβ<sub>17-21</sub>, or acceptable substitutions thereof. Accordingly, in preferred embodiments, Xaa<sub>1</sub> is a D-alanine structure or a D-leucine structure, Xaa<sub>2</sub> is a D-valine structure, Xaa<sub>3</sub> is a D-phenylalanine structure, a D-tyrosine structure or a D-iodotyrosine structure, Xaa<sub>4</sub> is a D-phenylalanine structure, a D-tyrosine structure or a D-iodotyrosine structure, and Xaa<sub>5</sub> is a D-alanine structure or a D-leucine structure.

15 In another preferred embodiment, Xaa<sub>1</sub>, Xaa<sub>2</sub>, Xaa<sub>3</sub> and Xaa<sub>4</sub> of formula (III) are selected based on the retro-inverso isomer of Aβ<sub>17-20</sub>, or acceptable substitutions thereof. Accordingly, in preferred embodiments, Xaa<sub>1</sub> is a D-alanine structure, a D-leucine structure or a D-phenylalanine structure, Xaa<sub>2</sub> is a D-phenylalanine structure, a D-tyrosine structure or a D-iodotyrosine structure, Xaa<sub>3</sub> is a D-phenylalanine structure, a D-tyrosine structure or a D-iodotyrosine structure and Xaa<sub>4</sub> is a D-valine structure or a D-leucine structure.

20 In another preferred embodiment, Xaa<sub>1</sub>, Xaa<sub>2</sub>, Xaa<sub>3</sub>, Xaa<sub>4</sub> and Xaa<sub>5</sub> of formula (IV) are selected based on the retroinverso isomer of Aβ<sub>17-21</sub>, or acceptable substitutions thereof. Accordingly, in preferred embodiments, Xaa<sub>1</sub> is a D-alanine structure, a D-leucine structure or a D-phenylalanine structure, Xaa<sub>2</sub> is a D-phenylalanine structure, a D-tyrosine structure or a D-iodotyrosine structure, Xaa<sub>3</sub> is a D-phenylalanine structure, a D-tyrosine structure or a D-iodotyrosine structure, Xaa<sub>4</sub> is a D-valine structure or a D-leucine structure and Xaa<sub>5</sub> is a D-leucine structure.

25 In one embodiment of the compounds of formulas (III) and/or (IV), A is present and comprises a cyclic, heterocyclic, polycyclic or branched alkyl group. In another embodiment of the compounds of formulas (III) and/or (IV), B is present and comprises an amide group, an alkyl amide group, an aryl amide group or a hydroxy group. In yet

- 18 -

another embodiment of the compounds of formulas (III) and/or (IV), both A and B are present.

In preferred specific embodiments, the invention provides a  $\beta$ -amyloid modulator compound comprising a peptidic structure selected from the group consisting of D-Leu-D-Val-D-Phe-D-Phe (SEQ ID NO: 9), D-Leu-D-Val-D-Phe-phenethylamide (SEQ ID NO: 10), D-Leu-D-Val-D-Tyr-D-Phe (SEQ ID NO: 11), D-Leu-D-Val-D-IodoTyr-D-Phe (SEQ ID NO: 12), D-Leu-D-Val-D-Phe-D-Tyr (SEQ ID NO: 13), D-Leu-D-Val-D-Phe-D-IodoTyr (SEQ ID NO: 14), D-Leu-D-Val-D-Phe-D-Ala (SEQ ID NO: 15), D-Leu-D-Val-D-Phe-D-Phe-D-Ala (SEQ ID NO: 16), D-Ala-D-Val-D-Phe-D-Phe-D-Leu (SEQ ID NO: 17), D-Leu-D-Val-D-Tyr-D-Phe-D-Ala (SEQ ID NO: 18), D-Leu-D-Val-D-IodoTyr-D-Phe-D-Ala (SEQ ID NO: 19), D-Leu-D-Val-D-Phe-D-Tyr-D-Ala (SEQ ID NO: 20), D-Leu-D-Val-D-Phe-D-IodoTyr-D-Ala (SEQ ID NO: 21), D-Phe-D-Phe-D-Val-D-Leu (SEQ ID NO: 22), D-Ala-D-Phe-D-Phe-D-Val (SEQ ID NO: 23), D-Ala-D-Phe-D-Phe-D-Val-D-Leu (SEQ ID NO: 4), D-Ala-D-Phe-D-Phe-D-Leu-D-Leu (SEQ ID NO: 5), D-Leu-D-Phe-D-Phe-D-Val-D-Leu (SEQ ID NO: 6), D-Phe-D-Phe-D-Phe-D-Val-D-Leu (SEQ ID NO: 7), D-Phe-D-Phe-D-Phe-D-Leu-D-Val (SEQ ID NO: 24), D-Phe-D-Phe-D-Phe-D-Phe-D-Leu (SEQ ID NO: 25) and D-Ala-D-Phe-D-Phe-D-Phe-D-Leu (SEQ ID NO: 26). Any of the aforementioned specific peptidic structures can be amino-terminally and/or carboxy-terminally modified and described further in subsections II and/or III below.

Particularly preferred modulators comprise D-amino acid peptide amides designed based on the retro-inverso isomer of A $\beta$ <sub>17-21</sub>, or acceptable substitutions thereof, including compounds selected from the group consisting of D-Ala-D-Phe-D-Phe-D-Val-D-Leu-amide (SEQ ID NO: 4; C-terminal amide), D-Ala-D-Phe-D-Phe-D-Leu-D-Leu-amide (SEQ ID NO: 5; C-terminal amide), D-Leu-D-Phe-D-Phe-D-Val-D-Leu-amide (SEQ ID NO: 6; C-terminal amide) and D-Phe-D-Phe-D-Phe-D-Val-D-Leu-amide (SEQ ID NO: 7; C-terminal amide), D-Phe-D-Phe-D-Phe-D-Leu-D-Val-amide (SEQ ID NO: 24; C-terminal amide), D-Phe-D-Phe-D-Phe-D-Phe-D-Leu-amide (SEQ ID NO: 25; C-terminal amide) and D-Ala-D-Phe-D-Phe-D-Phe-D-Leu-amide (SEQ ID NO: 26; C-terminal amide).

The D-amino acid peptidic structures of the modulators of the invention are further intended to include other peptide modifications, including analogues, derivatives and mimetics, that retain the ability of the modulator to alter natural  $\beta$ -AP aggregation as described herein. For example, a D-amino acid peptidic structure of a modulator of the invention may be further modified to increase its stability, bioavailability, solubility,

- 19 -

etc. The terms "analogue", "derivative" and "mimetic" as used herein are intended to include molecules which mimic the chemical structure of a D-peptidic structure and retain the functional properties of the D-peptidic structure. Approaches to designing peptide analogs, derivatives and mimetics are known in the art. For example, see

- 5 Farmer, P.S. in Drug Design (E.J. Ariens, ed.) Academic Press, New York, 1980, vol. 10, pp. 119-143; Ball, J.B. and Alewood, P.F. (1990) *J. Mol. Recognition* 3:55; Morgan, B.A. and Gainor, J.A. (1989) *Ann. Rep. Med. Chem.* 24:243; and Freidinger, R.M. (1989) *Trends Pharmacol. Sci.* 10:270. See also Sawyer, T.K. (1995) "Peptidomimetic Design and Chemical Approaches to Peptide Metabolism" in Taylor, M.D. and Amidon, G.L. (eds.) *Peptide-Based Drug Design: Controlling Transport and Metabolism*,  
10 Chapter 17; Smith, A.B. 3rd, *et al.* (1995) *J. Am. Chem. Soc.* 117:11113-11123; Smith, A.B. 3rd, *et al.* (1994) *J. Am. Chem. Soc.* 116:9947-9962; and Hirschman, R., *et al.* (1993) *J. Am. Chem. Soc.* 115:12550-12568.

- As used herein, a "derivative" of a compound X (*e.g.*, a peptide or amino acid)  
15 refers to a form of X in which one or more reaction groups on the compound have been derivatized with a substituent group. Examples of peptide derivatives include peptides in which an amino acid side chain, the peptide backbone, or the amino- or carboxy-terminus has been derivatized (*e.g.*, peptidic compounds with methylated amide linkages). As used herein an "analogue" of a compound X refers to a compound which  
20 retains chemical structures of X necessary for functional activity of X yet which also contains certain chemical structures which differ from X. An examples of an analogue of a naturally-occurring peptide is a peptide which includes one or more non-naturally-occurring amino acids. As used herein, a "mimetic" of a compound X refers to a compound in which chemical structures of X necessary for functional activity of X have  
25 been replaced with other chemical structures which mimic the conformation of X. Examples of peptidomimetics include peptidic compounds in which the peptide backbone is substituted with one or more benzodiazepine molecules (see *e.g.*, James, G.L. *et al.* (1993) *Science* 260:1937-1942).

- Analogues of the modulator compounds of the invention are intended to include  
30 compounds in which one or more D-amino acids of the peptidic structure are substituted with a homologous amino acid such that the properties of the original modulator are maintained. Preferably conservative amino acid substitutions are made at one or more amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain.  
35 Families of amino acid residues having similar side chains have been defined in the art,

- 20 -

including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan),  $\beta$ -branched  
5 side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Non-limiting examples of homologous substitutions that can be made in the peptidic structures of the modulators of the invention include substitution of D-phenylalanine with D-tyrosine, D-pyridylalanine or D-homophenylalanine, substitution of D-leucine with D-valine or other natural or non-  
10 natural amino acid having an aliphatic side chain and/or substitution of D-valine with D-leucine or other natural or non-natural amino acid having an aliphatic side chain.

The term mimetic, and in particular, peptidomimetic, is intended to include isosteres. The term "isostere" as used herein is intended to include a chemical structure that can be substituted for a second chemical structure because the steric conformation  
15 of the first structure fits a binding site specific for the second structure. The term specifically includes peptide back-bone modifications (*i.e.*, amide bond mimetics) well known to those skilled in the art. Such modifications include modifications of the amide nitrogen, the  $\alpha$ -carbon, amide carbonyl, complete replacement of the amide bond, extensions, deletions or backbone crosslinks. Several peptide backbone modifications  
20 are known, including  $\psi[\text{CH}_2\text{S}]$ ,  $\psi[\text{CH}_2\text{NH}]$ ,  $\psi[\text{CSNH}_2]$ ,  $\psi[\text{NHCO}]$ ,  $\psi[\text{COCH}_2]$ , and  $\psi[(\text{E}) \text{ or } (\text{Z}) \text{CH}=\text{CH}]$ . In the nomenclature used above,  $\psi$  indicates the absence of an amide bond. The structure that replaces the amide group is specified within the brackets.

Other possible modifications include an N-alkyl (or aryl) substitution ( $\psi$   
25  $[\text{CONR}]$ ), or backbone crosslinking to construct lactams and other cyclic structures. Other derivatives of the modulator compounds of the invention include C-terminal hydroxymethyl derivatives, *O*-modified derivatives (*e.g.*, C-terminal hydroxymethyl benzyl ether), N-terminally modified derivatives including substituted amides such as alkylamides and hydrazides and compounds in which a C-terminal phenylalanine  
30 residue is replaced with a phenethylamide analogue (*e.g.*, Val-Phe-phenethylamide as an analogue of the tripeptide Val-Phe-Phe).

The modulator compounds of the invention can be incorporated into pharmaceutical compositions (described further in subsection V below) and can be used in detection and treatment methods as described further in subsection VI below.

35

## II. Modifying Groups

In certain embodiments of the modulator compounds of the invention, a D-amino acid peptidic structure (such as an A $\beta$  derived peptide, or an A $\beta$  aggregation core domain, or an amino acid sequence corresponding to a rearranged A $\beta$  aggregation core domain) is coupled directly or indirectly to at least one modifying group (abbreviated as MG). The term "modifying group" is intended to include structures that are directly attached to the D-amino acid peptidic structure (*e.g.*, by covalent coupling), as well as those that are indirectly attached to the peptidic structure (*e.g.*, by a stable non-covalent association or by covalent coupling to additional amino acid residues, or mimetics, analogues or derivatives thereof, which may flank the A $\beta$ -derived D-amino acid peptidic structure). For example, the modifying group can be coupled to the amino-terminus or carboxy-terminus of an A $\beta$ -derived D-amino acid peptidic structure, or to a peptidic or peptidomimetic region flanking the core domain. Alternatively, the modifying group can be coupled to a side chain of at least one D-amino acid residue of an A $\beta$ -derived D-amino acid peptidic structure, or to a peptidic or peptidomimetic region flanking the core domain (*e.g.*, through the epsilon amino group of a lysyl residue(s), through the carboxyl group of an aspartic acid residue(s) or a glutamic acid residue(s), through a hydroxy group of a tyrosyl residue(s), a serine residue(s) or a threonine residue(s) or other suitable reactive group on an amino acid side chain). Modifying groups covalently coupled to the D-amino acid peptidic structure can be attached by means and using methods well known in the art for linking chemical structures, including, for example, amide, alkylamino, carbamate, urea or ester bonds.

The term "modifying group" is intended to include groups that are not naturally coupled to natural A $\beta$  peptides in their native form. Accordingly, the term "modifying group" is not intended to include hydrogen. The modifying group(s) is selected such that the modulator compound alters, and preferably inhibits, aggregation of natural  $\beta$ -amyloid peptides when contacted with the natural  $\beta$ -amyloid peptides or inhibits the neurotoxicity of natural  $\beta$ -amyloid peptides when contacted with the natural  $\beta$ -amyloid peptides. Although not intending to be limited by mechanism, in embodiments where the modulator comprises a modifying group(s), the modifying group(s) is thought to function as a key pharmacophore that enhances the ability of the modulator to disrupt A $\beta$  polymerization.

In a preferred embodiment, the modifying group(s) comprises a cyclic, heterocyclic, polycyclic or branched alkyl group. The term "cyclic group", as used herein, is intended to include cyclic saturated or unsaturated (*i.e.*, aromatic) group



- 22 -

having from about 3 to 10, preferably about 4 to 8, and more preferably about 5 to 7, carbon atoms. Exemplary cyclic groups include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and cyclooctyl. Cyclic groups may be unsubstituted or substituted at one or more ring positions. Thus, a cyclic group may be substituted with, *e.g.*, halogens, alkyls, cycloalkyls, alkenyls, alkynyls, aryls, heterocycles, hydroxyls, aminos, nitros, thiols amines, imines, amides, phosphonates, phosphines, carbonyls, carboxyls, silyls, ethers, thioethers, sulfonyls, sulfonates, selenoethers, ketones, aldehydes, esters, -CF<sub>3</sub>, -CN, or the like.

The term "heterocyclic group" is intended to include cyclic saturated or unsaturated (*i.e.*, aromatic) group having from about 3 to 10, preferably about 4 to 8, and more preferably about 5 to 7, carbon atoms, wherein the ring structure includes about one to four heteroatoms. Heterocyclic groups include pyrrolidine, oxolane, thiolane, imidazole, oxazole, piperidine, piperazine, morpholine and pyridine. The heterocyclic ring can be substituted at one or more positions with such substituents as, for example, halogens, alkyls, cycloalkyls, alkenyls, alkynyls, aryls, other heterocycles, hydroxyl, amino, nitro, thiol, amines, imines, amides, phosphonates, phosphines, carbonyls, carboxyls, silyls, ethers, thioethers, sulfonyls, selenoethers, ketones, aldehydes, esters, -CF<sub>3</sub>, -CN, or the like. Heterocycles may also be bridged or fused to other cyclic groups as described below.

The term "polycyclic group" as used herein is intended to refer to two or more saturated or unsaturated (*i.e.*, aromatic) cyclic rings in which two or more carbons are common to two adjoining rings, *e.g.*, the rings are "fused rings". Rings that are joined through non-adjacent atoms are termed "bridged" rings. Each of the rings of the polycyclic group can be substituted with such substituents as described above, as for example, halogens, alkyls, cycloalkyls, alkenyls, alkynyls, hydroxyl, amino, nitro, thiol, amines, imines, amides, phosphonates, phosphines, carbonyls, carboxyls, silyls, ethers, thioethers, sulfonyls, selenoethers, ketones, aldehydes, esters, -CF<sub>3</sub>, -CN, or the like.

A preferred polycyclic group is a group containing a cis-decalin structure. Although not intending to be limited by mechanism, it is thought that the "bent" conformation conferred on a modifying group by the presence of a cis-decalin structure contributes to the efficacy of the modifying group in disrupting A $\beta$  polymerization. Accordingly, other structures which mimic the "bent" configuration of the cis-decalin structure can also be used as modifying groups. An example of a cis-decalin containing structure that can be used as a modifying group is a cholanoyl structure, such as a cholyl group. For example, a modulator compound can be modified at its amino terminus with

- 23 -

a cholyl group by reacting the aggregation core domain with cholic acid, a bile acid. Moreover, a modulator compound can be modified at its carboxy terminus with a cholyl group according to methods known in the art (see *e.g.*, Wess, G. *et al.* (1993) *Tetrahedron Letters*, 34:817-822; Wess, G. *et al.* (1992) *Tetrahedron Letters* 33:195-198; and Kramer, W. *et al.* (1992) *J. Biol. Chem.* 267:18598-18604). Cholyl derivatives and analogues can also be used as modifying groups. For example, a preferred cholyl derivative is Aic (3-(*O*-aminoethyl-*iso*)-cholyl), which has a free amino group that can be used to further modify the modulator compound (*e.g.*, a chelation group for <sup>99m</sup>Tc can be introduced through the free amino group of Aic). As used herein, the term "cholanoyl structure" is intended to include the cholyl group and derivatives and analogues thereof, in particular those which retain a four-ring *cis*-decalin configuration. Examples of cholanoyl structures include groups derived from other bile acids, such as deoxycholic acid, lithocholic acid, ursodeoxycholic acid, chenodeoxycholic acid and hyodeoxycholic acid, as well as other related structures such as cholanic acid, bufalin and resibufogenin (although the latter two compounds are not preferred for use as a modifying group). Another example of a *cis*-decalin containing compound is 5 $\beta$ -cholestan-3 $\alpha$ -ol (the *cis*-decalin isomer of (+)-dihydrocholesterol). For further description of bile acid and steroid structure and nomenclature, see Nes, W.R. and McKean, M.L. *Biochemistry of Steroids and Other Isopentanoids*, University Park Press, Baltimore, MD, Chapter 2.

In addition to *cis*-decalin containing groups, other polycyclic groups may be used as modifying groups. For example, modifying groups derived from steroids or  $\beta$ -lactams may be suitable modifying groups. In one embodiment, the modifying group is a "biotinyl structure", which includes biotinyl groups and analogues and derivatives thereof (such as a 2-iminobiotinyl group). In another embodiment, the modifying group can comprise a "fluorescein-containing group", such as a group derived from reacting an A $\beta$ -derived peptidic structure with 5-(and 6-)-carboxyfluorescein, succinimidyl ester or fluorescein isothiocyanate. In various other embodiments, the modifying group(s) can comprise an *N*-acetylneuraminyl group, a *trans*-4-cotininecarboxyl group, a 2-imino-1-imidazolidineacetyl group, an (*S*)-(-)-indoline-2-carboxyl group, a (-)-menthoxyacetyl group, a 2-norbornaneacetyl group, a  $\gamma$ -oxo-5-acenaphthenebutyryl, a (-)-2-oxo-4-thiazolidinecarboxyl group, a tetrahydro-3-furoyl group, a 2-iminobiotinyl group, a diethylenetriaminepentaacetyl group, a 4-morpholinecarbonyl group, a 2-thiopheneacetyl group or a 2-thiophenesulfonyl group.

- 24 -

In addition to the cyclic, heterocyclic and polycyclic groups discussed above, other types of modifying groups can be used in a modulator of the invention. For example, hydrophobic groups and branched alkyl groups may be suitable modifying groups. Examples include acetyl groups, phenylacetyl groups, phenylacetyl groups, 5 diphenylacetyl groups, triphenylacetyl groups, isobutanoyl groups, 4-methylvaleryl groups, *trans*-cinnamoyl groups, butanoyl groups and 1-adamantanecarbonyl groups.

Yet another type of modifying group is a compound that contains a non-natural amino acid that acts as a beta-turn mimetic, such as a dibenzofuran-based amino acid described in Tsang, K.Y. et al. (1994) *J. Am. Chem. Soc.* 116:3988-4005; Diaz, H and 10 Kelly, J.W. (1991) *Tetrahedron Letters* 41:5725-5728; and Diaz, H et al. (1992) *J. Am. Chem. Soc.* 114:8316-8318. An example of such a modifying group is a peptide-aminoethyldibenzofuranyl-propionic acid (Adp) group (e.g., DDIIL-Adp) (SEQ ID NO: 31). This type of modifying group further can comprise one or more N-methyl peptide bonds to introduce additional steric hindrance to the aggregation of natural  $\beta$ -AP when 15 compounds of this type interact with natural  $\beta$ -AP.

- 25 -

Non-limiting examples of suitable modifying groups, with their corresponding modifying reagents, are listed below:

<u>Modifying Group</u>	<u>Modifying Reagent</u>
Cholyl-	Cholic acid
Lithocholyl-	Lithocholic acid
Hyodeoxycholyl-	Hyodeoxycholic acid
Chenodeoxycholyl-	Chenodeoxycholic acid
Ursodeoxycholyl-	Ursodeoxycholic acid
3-Hydroxycinnamoyl-	3-Hydroxycinnamic acid
4-Hydroxycinnamoyl-	4-Hydroxycinnamic acid
2-Hydroxycinnamoyl-	2-Hydroxycinnamic acid
3-Hydroxy-4-methoxycinnamoyl-	3-Hydroxy-4-methoxycinnamic acid
4-Hydroxy-3-methoxycinnamoyl-	4-Hydroxy-3-methoxycinnamic acid
2-Carboxycinnamoyl-	2-Carboxycinnamic acid
3-Formylbenzoyl	3-Carboxybenzaldehyde
4-Formylbenzoyl	4-Carboxybenzaldehyde
3,4,-Dihydroxyhydrocinnamoyl-	3,4,-Dihydroxyhydrocinnamic acid
3,7-Dihydroxy-2-naphthoyl-	3,7-Dihydroxy-2-naphthoic acid
4-Formylcinnamoyl-	4-Formylcinnamic acid
2-Formylphenoxyacetyl-	2-Formylphenoxyacetic acid
8-Formyl-1-naphthoyl	1,8-naphthaldehydic acid
4-(hydroxymethyl)benzoyl-	4-(hydroxymethyl)benzoic acid
4-Hydroxyphenylacetyl-	4-Hydroxyphenylacetic acid
3-Hydroxybenzoyl-	3-Hydroxybenzoic acid
4-Hydroxybenzoyl-	4-Hydroxybenzoic acid
5-Hydantoinacetyl-	5-Hydantoinacetic acid
L-Hydroorotyl-	L-Hydroorotic acid
4-Methylvaleryl-	4-Methylvaleric acid
2,4-Dihydroxybenzoyl-	2,4-Dihydroxybenzoic acid
3,4-Dihydroxycinnamoyl-	3,4-Dihydroxycinnamic acid
3,5-Dihydroxy-2-naphthoyl-	3,5-Dihydroxy-2-naphthoic acid
3-Benzoylpropanoyl-	3-Benzoylpropanoic acid
<i>trans</i> -Cinnamoyl-	<i>trans</i> -Cinnamic acid
Phenylacetyl-	Phenylacetic acid
Diphenylacetyl-	Diphenylacetic acid
Triphenylacetyl-	Triphenylacetic acid
2-Hydroxyphenylacetyl-	2-Hydroxyphenylacetic acid
3-Hydroxyphenylacetyl-	3-Hydroxyphenylacetic acid
4-Hydroxyphenylacetyl-	4-Hydroxyphenylacetic acid
(±)-Mandelyl-	(±)-Mandelic acid

- 26 -

(±)-2,4-Dihydroxy-3,3-dimethylbutanoyl	(±)-Pantolactone
Butanoyl-	Butanoic anhydride
Isobutanoyl-	Isobutanoic anhydride
Hexanoyl-	Hexanoic anhydride
Propionyl-	Propionic anhydride
3-Hydroxybutyroyl	β-Butyrolactone
4-Hydroxybutyroyl	γ-Butyrolactone
3-Hydroxypropionoyl	β-Propiolactone
2,4-Dihydroxybutyroyl	α-Hydroxy-β-Butyrolactone
1-Adamantanecarbonyl-	1-Adamantanecarbonic acid
Glycolyl-	Glycolic acid
DL-3-(4-hydroxyphenyl)lactyl-	DL-3-(4-hydroxyphenyl)lactic acid
3-(2-Hydroxyphenyl)propionyl-	3-(2-Hydroxyphenyl)propionic acid
4-(2-Hydroxyphenyl)propionyl-	4-(2-Hydroxyphenyl)propionic acid
D-3-Phenyllactyl-	D-3-Phenyllactic acid
Hydrocinnamoyl-	Hydrocinnamic acid
3-(4-Hydroxyphenyl)propionyl-	3-(4-Hydroxyphenyl)propionic acid
L-3-Phenyllactyl-	L-3-Phenyllactic acid
4-methylvaleryl	4-methylvaleric acid
3-pyridylacetyl	3-pyridylacetic acid
4-pyridylacetyl	4-pyridylacetic acid
Isonicotinoyl	
4-quinolinecarboxyl	4-quinolinecarboxylic acid
1-isoquinolinecarboxyl	1-isoquinolinecarboxylic acid
3-isoquinolinecarboxyl	3-isoquinolinecarboxylic acid

Preferred modifying groups include cis-decalin-containing groups, biotin-containing groups, fluorescein-containing groups, a diethylene-triaminepentaacetyl group, a (-)-menthoxyacetyl group, an N-acetylneuraminyl group, a phenylacetyl group, a diphenylacetyl group, a triphenylacetyl group, an isobutanoyl group, a 4-methylvaleryl group, a 3-hydroxyphenylacetyl group, a 2-hydroxyphenylacetyl group, a 3,5-dihydroxy-2-naphthoyl group, a 3,4-dihydroxycinnamoyl group, a (±)-mandelyl group, a (±)-mandelyl-(±)-mandelyl group, a glycolyl group, a benzoylpropanoyl group and a 2,4-dihydroxybenzoyl group.

### III. Additional Chemical Modifications of Aβ Modulators

A β-amyloid modulator compound of the invention can be further modified to alter the specific properties of the compound while retaining the ability of the compound to alter Aβ aggregation and inhibit Aβ neurotoxicity. For example, in one embodiment, the compound is further modified to alter a pharmacokinetic property of the compound,

- 27 -

such as *in vivo* stability or half-life. In another embodiment, the compound is further modified to label the compound with a detectable substance. In yet another embodiment, the compound is further modified to couple the compound to an additional therapeutic moiety. Schematically, a modulator of the invention comprising a D-amino acid A $\beta$  aggregation core domain coupled directly or indirectly to at least one modifying group can be illustrated as MG-ACD, whereas this compound which has been further modified to alter the properties of the modulator can be illustrated as MG-ACD-CM, wherein CM represents an additional chemical modification.

To further chemically modify the compound, such as to alter the pharmacokinetic properties of the compound, reactive groups can be derivatized. For example, when the modifying group is attached to the amino-terminal end of the aggregation core domain, the carboxy-terminal end of the compound can be further modified. Preferred C-terminal modifications include those which reduce the ability of the compound to act as a substrate for carboxypeptidases. Examples of preferred C-terminal modifiers include an amide group (*i.e.*, a peptide amide), an alkyl or aryl amide group (*e.g.*, an ethylamide group or a phenethylamide group) a hydroxy group (*i.e.*, a peptide alcohol) and various non-natural amino acids, such as D-amino acids and  $\beta$ -alanine. Alternatively, when the modifying group is attached to the carboxy-terminal end of the aggregation core domain, the amino-terminal end of the compound can be further modified, for example, to reduce the ability of the compound to act as a substrate for aminopeptidases.

A modulator compound can be further modified to label the compound by reacting the compound with a detectable substance. Suitable detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase,  $\beta$ -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include  $^{14}\text{C}$ ,  $^{123}\text{I}$ ,  $^{124}\text{I}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{99\text{m}}\text{Tc}$ ,  $^{35}\text{S}$  or  $^3\text{H}$ . In a preferred embodiment, a modulator compound is radioactively labeled with  $^{14}\text{C}$ , either by incorporation of  $^{14}\text{C}$  into the modifying group or one or more amino acid structures in the modulator compound. Labeled modulator compounds can be used to assess the *in vivo* pharmacokinetics of the compounds, as well as to detect A $\beta$

- 28 -

aggregation, for example for diagnostic purposes. A $\beta$  aggregation can be detected using a labeled modulator compound either *in vivo* or in an *in vitro* sample derived from a subject.

Preferably, for use as an *in vivo* diagnostic agent, a modulator compound of the invention is labeled with radioactive technetium or iodine. Accordingly, in one embodiment, the invention provides a modulator compound labeled with technetium, preferably  $^{99m}\text{Tc}$ . Methods for labeling peptide compounds with technetium are known in the art (see e.g., U.S. Patent Nos. 5,443,815, 5,225,180 and 5,405,597, all by Dean *et al.*; Stepniak-Biniakiewicz, D., *et al.* (1992) *J. Med. Chem.* 35:274-279; Fritzberg, A.R., *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4025-4029; Baidoo, K.E., *et al.* (1990) *Cancer Res. Suppl.* 50:799s-803s; and Regan, L. and Smith, C.K. (1995) *Science* 270:980-982). A modifying group can be chosen that provides a site at which a chelation group for  $^{99m}\text{Tc}$  can be introduced, such as the Aic derivative of cholic acid, which has a free amino group. In another embodiment, the invention provides a modulator compound labeled with radioactive iodine. For example, a phenylalanine residue within the A $\beta$  sequence (such as Phe<sub>19</sub> or Phe<sub>20</sub>) can be substituted with radioactive iodotyrosyl. Any of the various isotopes of radioactive iodine can be incorporated to create a diagnostic agent. Preferably,  $^{123}\text{I}$  (half-life = 13.2 hours) is used for whole body scintigraphy,  $^{124}\text{I}$  (half life = 4 days) is used for positron emission tomography (PET),  $^{125}\text{I}$  (half life = 60 days) is used for metabolic turnover studies and  $^{131}\text{I}$  (half life = 8 days) is used for whole body counting and delayed low resolution imaging studies.

Furthermore, an additional modification of a modulator compound of the invention can serve to confer an additional therapeutic property on the compound. That is, the additional chemical modification can comprise an additional functional moiety. For example, a functional moiety which serves to break down or dissolve amyloid plaques can be coupled to the modulator compound. In this form, the MG-ACD portion of the modulator serves to target the compound to A $\beta$  peptides and disrupt the polymerization of the A $\beta$  peptides, whereas the additional functional moiety serves to break down or dissolve amyloid plaques after the compound has been targeted to these sites.

In an alternative chemical modification, a  $\beta$ -amyloid compound of the invention is prepared in a "prodrug" form, wherein the compound itself does not modulate A $\beta$  aggregation, but rather is capable of being transformed, upon metabolism *in vivo*, into a  $\beta$ -amyloid modulator compound as defined herein. For example, in this type of

- 29 -

compound, the modulating group can be present in a prodrug form that is capable of being converted upon metabolism into the form of an active modulating group. Such a prodrug form of a modifying group is referred to herein as a "secondary modifying group." A variety of strategies are known in the art for preparing peptide prodrugs that  
5 limit metabolism in order to optimize delivery of the active form of the peptide-based drug (see *e.g.*, Moss, J. (1995) in *Peptide-Based Drug Design: Controlling Transport and Metabolism*, Taylor, M.D. and Amidon, G.L. (eds), Chapter 18. Additionally strategies have been specifically tailored to achieving CNS delivery based on "sequential metabolism" (see *e.g.*, Bodor, N., *et al.* (1992) *Science* 257:1698-1700; Prokai, L., *et al.*  
10 (1994) *J. Am. Chem. Soc.* 116:2643-2644; Bodor, N. and Prokai, L. (1995) in *Peptide-Based Drug Design: Controlling Transport and Metabolism*, Taylor, M.D. and Amidon, G.L. (eds), Chapter 14. In one embodiment of a prodrug form of a modulator of the invention, the modifying group comprises an alkyl ester to facilitate blood-brain barrier permeability.

15

Modulator compounds of the invention can be prepared by standard techniques known in the art. The peptide component of a modulator can be synthesized using standard techniques such as those described in Bodansky, M. *Principles of Peptide Synthesis*, Springer Verlag, Berlin (1993) and Grant, G.A (ed.). *Synthetic Peptides: A*  
20 *User's Guide*, W.H. Freeman and Company, New York (1992). Automated peptide synthesizers are commercially available (*e.g.*, Advanced ChemTech Model 396; Milligen/ Biosearch 9600). Additionally, one or more modulating groups can be attached to the A $\beta$ -derived peptidic component (*e.g.*, an A $\beta$  aggregation core domain) by standard methods, for example using methods for reaction through an amino group (*e.g.*,  
25 the alpha-amino group at the amino-terminus of a peptide), a carboxyl group (*e.g.*, at the carboxy terminus of a peptide), a hydroxyl group (*e.g.*, on a tyrosine, serine or threonine residue) or other suitable reactive group on an amino acid side chain (see *e.g.*, Greene, T.W and Wuts, P.G.M. *Protective Groups in Organic Synthesis*, John Wiley and Sons, Inc., New York (1991). Exemplary syntheses of D-amino acid  $\beta$  amyloid modulator are  
30 described further in Example 1.

#### IV. Screening Assays

Another aspect of the invention pertains to a method for selecting a modulator of  $\beta$ -amyloid aggregation. In the method, a test compound is contacted with natural  $\beta$   
35 amyloid peptides, the aggregation of the natural  $\beta$ -AP is measured and a modulator is



- 30 -

selected based on the ability of the test compound to alter the aggregation of the natural  $\beta$ -AP (*e.g.*, inhibit or promote aggregation). In a preferred embodiment, the test compound is contacted with a molar excess amount of the natural  $\beta$ -AP. The amount and/or rate of natural  $\beta$ -AP aggregation in the presence of the test compound can be  
5 determined by a suitable assay indicative of  $\beta$ -AP aggregation, as described herein (see *e.g.*, Example 2).

In a preferred assay, the natural  $\beta$ -AP is dissolved in solution in the presence of the test compound and aggregation of the natural  $\beta$ -AP is assessed in a nucleation assay (see Example 2) by assessing the turbidity of the solution over time, as measured by the  
10 apparent absorbance of the solution at 405 nm (described further in Example 2; see also Jarrett *et al.* (1993) *Biochemistry* 32:4693-4697). In the absence of a  $\beta$ -amyloid modulator, the  $A_{405\text{nm}}$  of the solution typically stays relatively constant during a lag time in which the  $\beta$ -AP remains in solution, but then the  $A_{405\text{nm}}$  of the solution rapidly increases as the  $\beta$ -AP aggregates and comes out of solution, ultimately reaching a  
15 plateau level (*i.e.*, the  $A_{405\text{nm}}$  of the solution exhibits sigmoidal kinetics over time). In contrast, in the presence of a test compound that inhibits  $\beta$ -AP aggregation, the  $A_{405\text{nm}}$  of the solution is reduced compared to when the modulator is absent. Thus, in the presence of the inhibitory modulator, the solution may exhibit an increased lag time, a decreased slope of aggregation and/or a lower plateau level compared to when the  
20 modulator is absent. This method for selecting a modulator of  $\beta$ -amyloid polymerization can similarly be used to select modulators that promote  $\beta$ -AP aggregation. Thus, in the presence of a modulator that promotes  $\beta$ -AP aggregation, the  $A_{405\text{nm}}$  of the solution is increased compared to when the modulator is absent (*e.g.*, the solution may exhibit an decreased lag time, increase slope of aggregation and/or a higher  
25 plateau level compared to when the modulator is absent).

Another assay suitable for use in the screening method of the invention, a seeded extension assay, is also described further in Example 2. In this assay,  $\beta$ -AP monomer and an aggregated  $\beta$ -AP "seed" are combined, in the presence and absence of a test compound, and the amount of  $\beta$ -fibril formation is assayed based on enhanced emission  
30 of the dye Thioflavine T when contacted with  $\beta$ -AP fibrils. Moreover,  $\beta$ -AP aggregation can be assessed by electron microscopy (EM) of the  $\beta$ -AP preparation in the presence or absence of the modulator. For example,  $\beta$  amyloid fibril formation, which is detectable by EM, is reduced in the presence of a modulator that inhibits  $\beta$ -AP aggregation (*i.e.*, there is a reduced amount or number of  $\beta$ -fibrils in the presence of the  
35 modulator), whereas  $\beta$  fibril formation is increased in the presence of a modulator that

- 31 -

promotes  $\beta$ -AP aggregation (*i.e.*, there is an increased amount or number of  $\beta$ -fibrils in the presence of the modulator).

Another preferred assay for use in the screening method of the invention to select suitable modulators is the neurotoxicity assay described in Example 3. Compounds are selected which inhibit the formation of neurotoxic A $\beta$  aggregates and/or which inhibit the neurotoxicity of preformed A $\beta$  fibrils. This neurotoxicity assay is considered to be predictive of neurotoxicity *in vivo*. Accordingly, inhibitory activity of a modulator compound in the *in vitro* neurotoxicity assay is predictive of similar inhibitory activity of the compound for neurotoxicity *in vivo*.

#### V. Pharmaceutical Compositions

Another aspect of the invention pertains to pharmaceutical compositions of the  $\beta$ -amyloid modulator compounds of the invention. In one embodiment, the composition includes a  $\beta$  amyloid modulator compound in a therapeutically or prophylactically effective amount sufficient to alter, and preferably inhibit, aggregation of natural  $\beta$ -amyloid peptides, and a pharmaceutically acceptable carrier. In another embodiment, the composition includes a  $\beta$  amyloid modulator compound in a therapeutically or prophylactically effective amount sufficient to inhibit the neurotoxicity of natural  $\beta$ -amyloid peptides, and a pharmaceutically acceptable carrier. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result, such as reduction or reversal of  $\beta$ -amyloid deposition and/or reduction or reversal of A $\beta$  neurotoxicity. A therapeutically effective amount of modulator may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the modulator to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. A therapeutically effective amount is also one in which any toxic or detrimental effects of the modulator are outweighed by the therapeutically beneficial effects. The potential neurotoxicity of the modulators of the invention can be assayed using the cell-based assay described in Example 6 and a therapeutically effective modulator can be selected which does not exhibit significant neurotoxicity. In a preferred embodiment, a therapeutically effective amount of a modulator is sufficient to alter, and preferably inhibit, aggregation of a molar excess amount of natural  $\beta$ -amyloid peptides. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result, such as preventing or inhibiting the rate of  $\beta$ -amyloid deposition and/or A $\beta$

- 32 -

neurotoxicity in a subject predisposed to  $\beta$ -amyloid deposition. A prophylactically effective amount can be determined as described above for the therapeutically effective amount. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the  
5 therapeutically effective amount.

One factor that may be considered when determining a therapeutically or prophylactically effective amount of a  $\beta$  amyloid modulator is the concentration of natural  $\beta$ -AP in a biological compartment of a subject, such as in the cerebrospinal fluid (CSF) of the subject. The concentration of natural  $\beta$ -AP in the CSF has been estimated  
10 at 3 nM (Schwartzman, (1994) *Proc. Natl. Acad. Sci. USA* 91:8368-8372). A non-limiting range for a therapeutically or prophylactically effective amounts of a  $\beta$  amyloid modulator is 0.01 nM-10  $\mu$ M. It is to be noted that dosage values may vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to  
15 the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

The amount of active compound in the composition may vary according to  
20 factors such as the disease state, age, sex, and weight of the individual, each of which may affect the amount of natural  $\beta$ -AP in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic  
25 situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required  
30 pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

- 33 -

As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. In one embodiment, the carrier is suitable for parenteral administration. Preferably, the carrier is suitable for administration into the central nervous system (*e.g.*, intraspinally or intracerebrally). Alternatively, the carrier can be suitable for intravenous, intraperitoneal or intramuscular administration. In another embodiment, the carrier is suitable for oral administration. Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, monostearate salts and gelatin. Moreover, the modulators can be administered in a time release formulation, for example in a composition which includes a slow release polymer. The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, polylactic acid and polylactic, polyglycolic copolymers (PLG). Many methods for the preparation of such formulations are patented or generally known to those skilled in the art.

- 34 -

Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*,  $\beta$ -amyloid modulator) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

A modulator compound of the invention can be formulated with one or more additional compounds that enhance the solubility of the modulator compound. Preferred compounds to be added to formulations to enhance the solubility of the modulators are cyclodextrin derivatives, preferably hydroxypropyl- $\gamma$ -cyclodextrin. Drug delivery vehicles containing a cyclodextrin derivative for delivery of peptides to the central nervous system are described in Bodor, N., *et al.* (1992) *Science* 257:1698-1700. For the  $\beta$ -amyloid modulators described herein, inclusion in the formulation of hydroxypropyl- $\gamma$ -cyclodextrin at a concentration 50-200 mM increases the aqueous solubility of the compounds. In addition to increased solubility, inclusion of a cyclodextrin derivative in the formulation may have other beneficial effects, since  $\beta$ -cyclodextrin itself has been reported to interact with the A $\beta$  peptide and inhibit fibril formation *in vitro* (Camilleri, P., *et al.* (1994) *FEBS Letters* 341:256-258. Accordingly, use of a modulator compound of the invention in combination with a cyclodextrin derivative may result in greater inhibition of A $\beta$  aggregation than use of the modulator alone. Chemical modifications of cyclodextrins are known in the art (Hanessian, S., *et al.* (1995) *J. Org. Chem.* 60:4786-4797). In addition to use as an additive in a pharmaceutical composition containing a modulator of the invention, cyclodextrin derivatives may also be useful as modifying groups and, accordingly, may also be covalently coupled to an A $\beta$  peptide compound to form a modulator compound of the invention.

Another preferred formulation for the modulator compounds to enhance brain uptake comprises the detergent Tween-80, polyethylene glycol (PEG) and ethanol in a saline solution. A non-limiting example of such a preferred formulation is 0.16% Tween-80, 1.3% PEG-3000 and 2% ethanol in saline.

In another embodiment, a pharmaceutical composition comprising a modulator of the invention is formulated such that the modulator is transported across the blood-

- 35 -

brain barrier (BBB). Various strategies known in the art for increasing transport across the BBB can be adapted to the modulators of the invention to thereby enhance transport of the modulators across the BBB (for reviews of such strategies, see *e.g.*, Pardridge, W.M. (1994) *Trends in Biotechnol.* 12:239-245; Van Bree, J.B. *et al.* (1993) *Pharm. World Sci.* 15:2-9; and Pardridge, W.M. *et al.* (1992) *Pharmacol. Toxicol.* 71:3-10). In one approach, the modulator is chemically modified to form a prodrug with enhanced transmembrane transport. Suitable chemical modifications include covalent linking of a fatty acid to the modulator through an amide or ester linkage (see *e.g.*, U.S. Patent 4,933,324 and PCT Publication WO 89/07938, both by Shashoua; U.S. Patent 5,284,876 by Hesse *et al.*; Toth, I. *et al.* (1994) *J. Drug Target.* 2:217-239; and Shashoua, V.E. *et al.* (1984) *J. Med. Chem.* 27:659-664) and glycyating the modulator (see *e.g.*, U.S. Patent 5,260,308 by Poduslo *et al.*). Also, N-acylamino acid derivatives may be used in a modulator to form a "lipidic" prodrug (see *e.g.*, 5,112,863 by Hashimoto *et al.*).

In another approach for enhancing transport across the BBB, a peptidic or peptidomimetic modulator is conjugated to a second peptide or protein, thereby forming a chimeric protein, wherein the second peptide or protein undergoes absorptive-mediated or receptor-mediated transcytosis through the BBB. Accordingly, by coupling the modulator to this second peptide or protein, the chimeric protein is transported across the BBB. The second peptide or protein can be a ligand for a brain capillary endothelial cell receptor ligand. For example, a preferred ligand is a monoclonal antibody that specifically binds to the transferrin receptor on brain capillary endothelial cells (see *e.g.*, U.S. Patents 5,182,107 and 5,154,924 and PCT Publications WO 93/10819 and WO 95/02421, all by Friden *et al.*). Other suitable peptides or proteins that can mediate transport across the BBB include histones (see *e.g.*, U.S. Patent 4,902,505 by Pardridge and Schimmel) and ligands such as biotin, folate, niacin, pantothenic acid, riboflavin, thiamin, pyridoxal and ascorbic acid (see *e.g.*, U.S. Patents 5,416,016 and 5,108,921, both by Heinsteins). Additionally, the glucose transporter GLUT-1 has been reported to transport glycopeptides (L-serinyl- $\beta$ -D-glucoside analogues of [Met5]enkephalin) across the BBB (Polt, R. *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:7114-1778). Accordingly, a modulator compound can be coupled to such a glycopeptide to target the modulator to the GLUT-1 glucose transporter. For example, a modulator compound which is modified at its amino terminus with the modifying group Aic (3-(*O*-aminoethyl-*iso*)-cholyl, a derivative of cholic acid having a free amino group) can be coupled to a glycopeptide through the amino group of Aic by standard methods. Chimeric proteins can be formed by recombinant DNA methods (*e.g.*, by formation of a chimeric gene

- 36 -

encoding a fusion protein) or by chemical crosslinking of the modulator to the second peptide or protein to form a chimeric protein. Numerous chemical crosslinking agents are known in the (*e.g.*, commercially available from Pierce, Rockford IL). A crosslinking agent can be chosen which allows for high yield coupling of the modulator  
5 to the second peptide or protein and for subsequent cleavage of the linker to release bioactive modulator. For example, a biotin-avidin-based linker system may be used.

In yet another approach for enhancing transport across the BBB, the modulator is encapsulated in a carrier vector which mediates transport across the BBB. For example, the modulator can be encapsulated in a liposome, such as a positively charged  
10 unilamellar liposome (see *e.g.*, PCT Publications WO 88/07851 and WO 88/07852, both by Faden) or in polymeric microspheres (see *e.g.*, U.S. Patent 5,413,797 by Khan *et al.*, U.S. Patent 5,271,961 by Mathiowitz *et al.* and 5,019,400 by Gombotz *et al.*). Moreover, the carrier vector can be modified to target it for transport across the BBB. For example, the carrier vector (*e.g.*, liposome) can be covalently modified with a  
15 molecule which is actively transported across the BBB or with a ligand for brain endothelial cell receptors, such as a monoclonal antibody that specifically binds to transferrin receptors (see *e.g.*, PCT Publications WO 91/04014 by Collins *et al.* and WO 94/02178 by Greig *et al.*).

In still another approach to enhancing transport of the modulator across the BBB,  
20 the modulator is coadministered with another agent which functions to permeabilize the BBB. Examples of such BBB "permeabilizers" include bradykinin and bradykinin agonists (see *e.g.*, U.S. Patent 5,112,596 by Malfroy-Camine) and peptidic compounds disclosed in U.S. Patent 5,268,164 by Kozarich *et al.*

Assays that measure the *in vitro* stability of the modulator compounds in  
25 cerebrospinal fluid (CSF) and the degree of brain uptake of the modulator compounds in animal models can be used as predictors of *in vivo* efficacy of the compounds. Suitable assays for measuring CSF stability and brain uptake are described in Examples 7 and 8, respectively.

A modulator compound of the invention can be formulated into a pharmaceutical  
30 composition wherein the modulator is the only active compound or, alternatively, the pharmaceutical composition can contain additional active compounds. For example, two or more modulator compounds may be used in combination. Moreover, a modulator compound of the invention can be combined with one or more other agents that have anti-amyloidogenic properties. For example, a modulator compound can be

- 37 -

combined with the non-specific cholinesterase inhibitor tacrine (COGNEX®, Parke-Davis).

In another embodiment, a pharmaceutical composition of the invention is provided as a packaged formulation. The packaged formulation may include a pharmaceutical composition of the invention in a container and printed instructions for administration of the composition for treating a subject having a disorder associated with  $\beta$ -amyloidosis, *e.g.* Alzheimer's disease.

#### VI. Methods of Using A $\beta$ Modulators

Another aspect of the invention pertains to methods for altering the aggregation or inhibiting the neurotoxicity of natural  $\beta$ -amyloid peptides. In the methods of the invention, natural  $\beta$  amyloid peptides are contacted with a  $\beta$  amyloid modulator such that the aggregation of the natural  $\beta$  amyloid peptides is altered or the neurotoxicity of the natural  $\beta$  amyloid peptides is inhibited. In a preferred embodiment, the modulator inhibits aggregation of the natural  $\beta$  amyloid peptides. In another embodiment, the modulator promotes aggregation of the natural  $\beta$  amyloid peptides. Preferably, aggregation of a molar excess amount of  $\beta$ -AP, relative to the amount of modulator, is altered upon contact with the modulator.

In the method of the invention, natural  $\beta$  amyloid peptides can be contacted with a modulator either *in vitro* or *in vivo*. Thus, the term "contacted with" is intended to encompass both incubation of a modulator with a natural  $\beta$ -AP preparation *in vitro* and delivery of the modulator to a site *in vivo* where natural  $\beta$ -AP is present. Since the modulator compound interacts with natural  $\beta$ -AP, the modulator compounds can be used to detect natural  $\beta$ -AP, either *in vitro* or *in vivo*. Accordingly, one use of the modulator compounds of the invention is as diagnostic agents to detect the presence of natural  $\beta$ -AP, either in a biological sample or *in vivo* in a subject. Furthermore, detection of natural  $\beta$ -AP utilizing a modulator compound of the invention further can be used to diagnose amyloidosis in a subject. Additionally, since the modulator compounds of the invention disrupt  $\beta$ -AP aggregation and inhibit  $\beta$ -AP neurotoxicity, the modulator compounds also are useful in the treatment of disorders associated with  $\beta$ -amyloidosis, either prophylactically or therapeutically. Accordingly, another use of the modulator compounds of the invention is as therapeutic agents to alter aggregation and/or neurotoxicity of natural  $\beta$ -AP.

In one embodiment, a modulator compound of the invention is used *in vitro*, for example to detect and quantitate natural  $\beta$ -AP in sample (*e.g.*, a sample of biological



- 38 -

fluid). To aid in detection, the modulator compound can be modified with a detectable substance. The source of natural  $\beta$ -AP used in the method can be, for example, a sample of cerebrospinal fluid (*e.g.*, from an AD patient, an adult susceptible to AD due to family history, or a normal adult). The natural  $\beta$ -AP sample is contacted with a modulator of the invention and aggregation of the  $\beta$ -AP is measured, such as by the assays described in Example 2. The degree of aggregation of the  $\beta$ -AP sample can then be compared to that of a control sample(s) of a known concentration of  $\beta$ -AP, similarly contacted with the modulator and the results can be used as an indication of whether a subject is susceptible to or has a disorder associated with  $\beta$ -amyloidosis. Moreover,  $\beta$ -AP can be detected by detecting a modulating group incorporated into the modulator. For example, modulators incorporating a biotin compound as described herein (*e.g.*, an amino-terminally biotinylated  $\beta$ -AP peptide) can be detected using a streptavidin or avidin probe which is labeled with a detectable substance (*e.g.*, an enzyme, such as peroxidase).

In another embodiment, a modulator compound of the invention is used *in vivo* to detect, and, if desired, quantitate, natural  $\beta$ -AP deposition in a subject, for example to aid in the diagnosis of  $\beta$  amyloidosis in the subject. To aid in detection, the modulator compound can be modified with a detectable substance, preferably  $^{99m}\text{Tc}$  or radioactive iodine (described further above), which can be detected *in vivo* in a subject. The labeled  $\beta$ -amyloid modulator compound is administered to the subject and, after sufficient time to allow accumulation of the modulator at sites of amyloid deposition, the labeled modulator compound is detected by standard imaging techniques. The radioactive signal generated by the labeled compound can be directly detected (*e.g.*, whole body counting), or alternatively, the radioactive signal can be converted into an image on an autoradiograph or on a computer screen to allow for imaging of amyloid deposits in the subject. Methods for imaging amyloidosis using radiolabeled proteins are known in the art. For example, serum amyloid P component (SAP), radiolabeled with either  $^{123}\text{I}$  or  $^{99m}\text{Tc}$ , has been used to image systemic amyloidosis (see *e.g.*, Hawkins, P.N. and Pepys, M.B. (1995) *Eur. J. Nucl. Med.* 22:595-599). Of the various isotopes of radioactive iodine, preferably  $^{123}\text{I}$  (half-life = 13.2 hours) is used for whole body scintigraphy,  $^{124}\text{I}$  (half life = 4 days) is used for positron emission tomography (PET),  $^{125}\text{I}$  (half life = 60 days) is used for metabolic turnover studies and  $^{131}\text{I}$  (half life = 8 days) is used for whole body counting and delayed low resolution imaging studies. Analogous to studies using radiolabeled SAP, a labeled modulator compound of the invention can be delivered to a subject by an appropriate route (*e.g.*, intravenously,

- 39 -

intraspinally, intracerebrally) in a single bolus, for example containing 100 µg of labeled compound carrying approximately 180 MBq of radioactivity.

The invention provides a method for detecting the presence or absence of natural β-amyloid peptides in a biological sample, comprising contacting a biological sample  
5 with a compound of the invention and detecting the compound bound to natural β-amyloid peptides to thereby detect the presence or absence of natural β-amyloid peptides in the biological sample. In one embodiment, the β-amyloid modulator compound and the biological sample are contacted *in vitro*. In another embodiment, the β-amyloid  
10 modulator compound is contacted with the biological sample by administering the β-amyloid modulator compound to a subject. For *in vivo* administration, preferably the compound is labeled with radioactive technetium or radioactive iodine.

The invention also provides a method for detecting natural β-amyloid peptides to facilitate diagnosis of a β-amyloidogenic disease, comprising contacting a biological  
15 sample with the compound of the invention and detecting the compound bound to natural β-amyloid peptides to facilitate diagnosis of a β-amyloidogenic disease. In one embodiment, the β-amyloid modulator compound and the biological sample are contacted *in vitro*. In another embodiment, the β-amyloid modulator compound is  
20 contacted with the biological sample by administering the β-amyloid modulator compound to a subject. For *in vivo* administration, preferably the compound is labeled with radioactive technetium or radioactive iodine. Preferably, use of the method facilitates diagnosis of Alzheimer's disease.

In another embodiment, the invention provides a method for altering natural β-AP aggregation or inhibiting β-AP neurotoxicity, which can be used prophylactically or  
therapeutically in the treatment or prevention of disorders associated with β amyloidosis,  
25 *e.g.*, Alzheimer's Disease. Modulator compounds of the invention can reduce the toxicity of natural β-AP aggregates to cultured neuronal cells. Moreover, the modulators also have the ability to reduce the neurotoxicity of preformed Aβ fibrils. Accordingly, the modulator compounds of the invention can be used to inhibit or  
30 prevent the formation of neurotoxic Aβ fibrils in subjects (*e.g.*, prophylactically in a subject predisposed to β-amyloid deposition) and can be used to reverse β-amyloidosis therapeutically in subjects already exhibiting β-amyloid deposition.

A modulator of the invention is contacted with natural β amyloid peptides present in a subject (*e.g.*, in the cerebrospinal fluid or cerebrum of the subject) to thereby  
alter the aggregation of the natural β-AP and/or inhibit the neurotoxicity of the natural  
35 β-APs. A modulator compound alone can be administered to the subject, or

- 40 -

alternatively, the modulator compound can be administered in combination with other therapeutically active agents (*e.g.*, as discussed above in subsection IV). When combination therapy is employed, the therapeutic agents can be coadministered in a single pharmaceutical composition, coadministered in separate pharmaceutical compositions or administered sequentially.

The modulator may be administered to a subject by any suitable route effective for inhibiting natural  $\beta$ -AP aggregation in the subject, although in a particularly preferred embodiment, the modulator is administered parenterally, most preferably to the central nervous system of the subject. Possible routes of CNS administration include intraspinal administration and intracerebral administration (*e.g.*, intracerebrovascular administration). Alternatively, the compound can be administered, for example, orally, intraperitoneally, intravenously or intramuscularly. For non-CNS administration routes, the compound can be administered in a formulation which allows for transport across the BBB. Certain modulators may be transported across the BBB without any additional further modification whereas others may need further modification as described above in subsection IV.

Suitable modes and devices for delivery of therapeutic compounds to the CNS of a subject are known in the art, including cerebrovascular reservoirs (*e.g.*, Ommaya or Rikker reservoirs; see *e.g.*, Raney, J.P. *et al.* (1988) *J. Neurosci. Nurs.* 20:23-29; Sundaresan, N. *et al.* (1989) *Oncology* 3:15-22), catheters for intrathecal delivery (*e.g.*, Port-a-Cath, Y-catheters and the like; see *e.g.*, Plummer, J.L. (1991) *Pain* 44:215-220; Yaksh, T.L. *et al.* (1986) *Pharmacol. Biochem. Behav.* 25:483-485), injectable intrathecal reservoirs (*e.g.*, Spinalgesic; see *e.g.*, Brazenor, G.A. (1987) *Neurosurgery* 21:484-491), implantable infusion pump systems (*e.g.*, Infusaid; see *e.g.*, Zierski, J. *et al.* (1988) *Acta Neurochem. Suppl.* 43:94-99; Kanoff, R.B. (1994) *J. Am. Osteopath. Assoc.* 94:487-493) and osmotic pumps (sold by Alza Corporation). A particularly preferred mode of administration is via an implantable, externally programmable infusion pump. Suitable infusion pump systems and reservoir systems are also described in U.S. Patent No. 5,368,562 by Blomquist and U.S. Patent No. 4,731,058 by Doan, developed by Pharmacia Deltec Inc.

The method of the invention for altering  $\beta$ -AP aggregation *in vivo*, and in particular for inhibiting  $\beta$ -AP aggregation, can be used therapeutically in diseases associated with abnormal  $\beta$  amyloid aggregation and deposition to thereby slow the rate of  $\beta$  amyloid deposition and/or lessen the degree of  $\beta$  amyloid deposition, thereby ameliorating the course of the disease. In a preferred embodiment, the method is used to

- 41 -

5 treat Alzheimer's disease (*e.g.*, sporadic or familial AD, including both individuals exhibiting symptoms of AD and individuals susceptible to familial AD). The method can also be used prophylactically or therapeutically to treat other clinical occurrences of  $\beta$  amyloid deposition, such as in Down's syndrome individuals and in patients with hereditary cerebral hemorrhage with amyloidosis-Dutch-type (HCHWA-D). While inhibition of  $\beta$ -AP aggregation is a preferred therapeutic method, modulators that promote  $\beta$ -AP aggregation may also be useful therapeutically by allowing for the sequestration of  $\beta$ -AP at sites that do not lead to neurological impairment.

10 Additionally, abnormal accumulation of  $\beta$ -amyloid precursor protein in muscle fibers has been implicated in the pathology of sporadic inclusion body myositis (IBM) (Askana, V. et al. (1996) *Proc. Natl. Acad. Sci. USA* 93:1314-1319; Askanas, V. et al. (1995) *Current Opinion in Rheumatology* 7:486-496). Accordingly, the modulators of the invention can be used prophylactically or therapeutically in the treatment of disorders in which  $\beta$ -AP, or APP, is abnormally deposited at non-neurological locations, 15 such as treatment of IBM by delivery of the modulators to muscle fibers.

This invention is further illustrated by the following examples which should not be construed as limiting. A modulator's ability to alter the aggregation of natural  $\beta$ -amyloid peptide and/or inhibit the neurotoxicity of natural  $\beta$ -amyloid peptide in the 20 assays described below are predictive of the modulator's ability to perform the same function *in vivo*. The contents of all references, patents and published patent applications cited throughout this application are hereby incorporated by reference.

25 **EXAMPLE 1: Preparation of  $\beta$ -amyloid Modulator Compounds**  
**Comprising D-Amino Acids**

$\beta$ -amyloid modulators comprising D-amino acids can be prepared by solid-phase peptide synthesis, for example using an N $\alpha$ -9-fluorenylmethyloxycarbonyl (Fmoc)-based protection strategy as follows. Starting with 2.5 mmoles of Fmoc-D-Val-Wang 30 resin, sequential additions of each amino acid are performed using a four-fold excess of protected amino acids, 1-hydroxybenzotriazole (HOBt) and diisopropyl carbodiimide (DIC). Recouplings are performed when necessary as determined by ninhydrin testing of the resin after coupling. Each synthesis cycle is minimally described by a three 35 minute deprotection (25 % piperidine/N-methyl-pyrrolidone (NMP)), a 15 minute

- 42 -

deprotection, five one minute NMP washes, a 60 minute coupling cycle, five NMP washes and a ninhydrin test. For N-terminal modification, an N-terminal modifying reagent is substituted for an Fmoc-D-amino acid and coupled to a 700 mg portion of the fully assembled peptide-resin by the above protocol. The peptide is removed from the resin by treatment with trifluoroacetic acid (TFA) (82.5 %), water (5 %), thioanisole (5 %), phenol (5 %), ethanedithiol (2.5 %) for two hours followed by precipitation of the peptide in cold ether. The solid is pelleted by centrifugation (2400 rpm x 10 min.), and the ether decanted. The solid is resuspended in ether, pelleted and decanted a second time. The solid is dissolved in 10 % acetic acid and lyophilized to dryness. For structural analysis, 60 mg of the solid is dissolved in 25 % acetonitrile (ACN) /0.1 % TFA and applied to a C18 reversed phase high performance liquid chromatography (HPLC) column.

Alternatively,  $\beta$ -amyloid modulators comprising D-amino acids can be prepared on an Advanced ChemTech Model 396 multiple peptide synthesizer using an automated protocol established by the manufacturer for 0.025 mmole scale synthesis. Double couplings are performed on all cycles using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/N,N-diisopropylethylamine (DIEA)/HOBt/Fmoc-D-amino acid in four-fold excess for 30 minutes followed by DIC/HOBt/Fmoc-D-amino acid in four-fold excess for 45 minutes. The peptide is deprotected and removed from the resin by treatment with TFA/water (95 %/5 %) for three hours and precipitated with ether as described above. The pellet is resuspended in 10 % acetic acid and lyophilized. The material is purified by a preparative HPLC using 15 %-40 % acetonitrile over 80 minutes on a Vydac C18 column (21 x 250 mm).

Various *N*-terminally modified  $\beta$ -amyloid modulator compounds can be synthesized using standard methods. Fully-protected resin-bound peptides are prepared as described above on Wang resin to eventually afford carboxyl terminal peptide acids. Small portions of each peptide resin (*e.g.*, 13-20  $\mu$ moles) are aliquoted into the wells of the reaction block of an Advanced ChemTech Model 396 Multiple Peptide Synthesizer. The *N*-terminal Fmoc protecting group of each sample is removed in the standard manner with 25% piperidine in NMP followed by extensive washing with NMP. The unprotected *N*-terminal  $\alpha$ -amino group of each peptide-resin sample can be modified using one of the following methods:

Method A, coupling of modifying reagents containing free carboxylic acid groups: The modifying reagent (five equivalents) is predissolved in NMP, DMSO or a mixture of these two solvents. HOBt and DIC (five equivalents of each reagent) are

- 43 -

added to the dissolved modifier and the resulting solution is added to one equivalent of free-amino peptide-resin. Coupling is allowed to proceed overnight, followed by washing. If a ninhydrin test on a small sample of peptide-resin shows that coupling is not complete, the coupling is repeated using 1-hydroxy-7-azabenzotriazole (HOAt) in place of HOBt.

Method B, coupling of modifying reagents obtained in preactivated forms: The modifying reagent (five equivalents) is predissolved in NMP, DMSO or a mixture of these two solvents and added to one equivalent of peptide-resin. Diisopropylethylamine (DIEA; six equivalents) is added to the suspension of activated modifier and peptide-resin. Coupling is allowed to proceed overnight, followed by washing. If a ninhydrin test on a small sample of peptide-resin shows that coupling is not complete, the coupling is repeated.

After the second coupling (if required) the *N*-terminally modified peptide-resins are dried at reduced pressure and cleaved from the resin with removal of side-chain protecting groups as described above. Analytical reversed-phase HPLC is used to confirm that a major product is present in the resulting crude peptides, which are purified using Millipore Sep-Pak cartridges or preparative reverse-phase HPLC. Mass spectrometry or high-field nuclear magnetic resonance spectrometry is used to confirm the presence of the desired compound in the product.

## **EXAMPLE 2:**

### **β-Amyloid Aggregation Assays**

The ability of β-amyloid modulator compounds to modulate (*e.g.*, inhibit or promote) the aggregation of natural β-AP when combined with the natural β-AP can be examined in one or both of the aggregation assays described below. Natural β-AP (β-AP<sub>1-40</sub>) for use in the aggregation assays is commercially available from Bachem (Torrance, CA).

#### **A. Nucleation Assay**

The nucleation assay is employed to determine the ability of test compounds to alter (*e.g.* inhibit) the early events in formation of β-AP fibers from monomeric β-AP. Characteristic of a nucleated polymerization mechanism, a lag time is observed prior to nucleation, after which the peptide rapidly forms fibers as reflected in a linear rise in turbidity. The time delay before polymerization of β-AP monomer can be quantified as well as the extent of formation of insoluble fiber by light scattering (turbidity).

- 44 -

Polymerization reaches equilibrium when the maximum turbidity reaches a plateau. The turbidity of a solution of natural  $\beta$ -AP in the absence or presence of various concentrations of a  $\beta$ -amyloid modulator compound is determined by measuring the apparent absorbance of the solution at 405nm ( $A_{405\text{ nm}}$ ) over time. The threshold of sensitivity for the measurement of turbidity is in the range of 15-20  $\mu\text{M}$   $\beta$ -AP. A decrease in turbidity over time in the presence of the modulator, as compared to the turbidity in the absence of the modulator, indicates that the modulator inhibits formation of  $\beta$ -AP fibers from monomeric  $\beta$ -AP. This assay can be performed using stirring or shaking to accelerate polymerization, thereby increasing the speed of the assay.

Moreover the assay can be adapted to a 96-well plate format to screen multiple compounds.

To perform the nucleation assay, first  $A\beta_{1-40}$  peptide is dissolved in HFIP (1,1,1,3,3,3-Hexafluoro-2-propanol; Aldrich 10,522-8) at a concentration of 2 mg peptide/ml and incubated at room temperature for 30 min. HFIP-solubilized peptide is sonicated in a waterbath sonicator for 5 min at highest setting, then evaporated to dryness under a stream of argon. The peptide film is resuspended in anhydrous dimethylsulfoxide (DMSO) at a concentration of 6.9 mg/ml (25x concentration), sonicated for 5 min as before, then filtered through a 0.2 micron nylon syringe filter (VWR cat. No. 28196-050). Test compounds are dissolved in DMSO at a 100x concentration. Four volumes of 25x  $A\beta_{1-40}$  peptide in DMSO are combined with one volume of test compound in DMSO in a glass vial, and mixed to produce a 1:1 molar ratio of  $A\beta$  peptide to test compound. For different molar ratios, test compounds are diluted with DMSO prior to addition to  $A\beta_{1-40}$ , in order to keep the final DMSO and  $A\beta_{1-40}$  concentrations constant. Control samples do not contain the test compound.

Ten microliters of the mixture is then added to the bottom of a well of a Corning Costar ultra low binding 96-well plate (Corning Costar, Cambridge MA; cat. No. 2500). Ninety microliters of water is added to the well, the plate is shaken on a rotary shaker at a constant speed at room temperature for 30 seconds, an additional 100  $\mu\text{l}$  of 2x PTL buffer (20 mM  $\text{NaH}_2\text{PO}_4$ , 300 mM  $\text{NaCl}$ , pH 7.4) is added to the well, the plate is reshaken for 30 seconds and a baseline ( $t=0$ ) turbidity reading is taken by measuring the apparent absorbance at 405 nm using a Bio-Rad Model 450 Microplate Reader. The plate is then returned to the shaker and shaken continuously for 5 hours. Turbidity readings are taken at 15 minute intervals.

$\beta$ -amyloid aggregation in the absence of any modulators results in enhanced turbidity of the natural  $\beta$ -AP solution (*i.e.*, an increase in the apparent absorbance at 405

- 45 -

nm over time). Accordingly, a solution including an effective inhibitory modulator compound exhibits reduced turbidity as compared to the control sample without the modulator compound (*i.e.*, less apparent absorbance at 405 nm over time as compared to the control sample).

- 5           Alternative to use of turbidity to quantitate  $\beta$ -amyloid aggregation, fluorescence of thioflavin T (Th-T) also can be used to quantitate  $\beta$ -amyloid aggregation in the nucleation assay (use of Th-T fluorescence for quantitating  $\beta$ -amyloid aggregation is described further below for the seeded extension assay).

10    B. Seeded Extension Assay

- The seeded extension assay can be employed to measure the rate of  $A\beta$  fiber formed in a solution of  $A\beta$  monomer following addition of polymeric  $A\beta$  fiber "seed". The ability of test compounds to prevent further deposition of monomeric  $A\beta$  to previously deposited amyloid is determined using a direct indicator of  $\beta$ -sheet formation  
15    using fluorescence. In contrast with the nucleation assay, the addition of seed provides immediate nucleation and continued growth of preformed fibrils without the need for continuous mixing, and thus results in the absence of a lag time before polymerization starts. Since this assay uses static polymerization conditions, the activity of positive compounds in the nucleation assay can be confirmed in this second assay under different  
20    conditions and with an additional probe of amyloid structure.

- In the seeded extension assay, monomeric  $A\beta_{1-40}$  is incubated in the presence of a "seed" nucleus (approximately ten mole percent of  $A\beta$  that has been previously allowed to polymerize under controlled static conditions). Samples of the solution are then diluted in thioflavin T (Th-T). The polymer-specific association of Th-T with  $A\beta$   
25    produces a fluorescent complex that allows the measurement of the extent of fibril formation (Levine, H. (1993) *Protein Science* 2:404-410). In particular, association of Th-T with aggregated  $\beta$ -AP, but not monomeric or loosely associated  $\beta$ -AP, gives rise to a new excitation (ex) maximum at 450 nm and an enhanced emission (em) at 482 nm, compared to the 385 nm (ex) and 445 nm (em) for the free dye. Small aliquots of the  
30    polymerization mixture contain sufficient fibril to be mixed with Th-T to allow the monitoring of the reaction mixture by repeated sampling. A linear growth curve is observed in the presence of excess monomer. The formation of thioflavin T responsive  $\beta$ -sheet fibrils parallels the increase in turbidity observed using the nucleation assay.

- A solution of  $A\beta$  monomer for use in the seeded extension assay is prepared by  
35    dissolving an appropriate quantity of  $A\beta_{1-40}$  peptide in 1/25 volume of



- 46 -

dimethylsulfoxide (DMSO), followed by water to 1/2 volume and 1/2 volume 2x PBS (10x PBS: NaCl 137 mM, KCl 2.7 mM Na<sub>2</sub>HPO<sub>4</sub> • 7H<sub>2</sub>O 4.3 mM, KH<sub>2</sub>PO<sub>4</sub> 1.4 mM pH 7.2) to a final concentration of 200  $\mu$ M. To prepare the stock seed, 1 ml of the A $\beta$  monomer preparation, is incubated for approximately 8 days at 37 °C and sheared sequentially through an 18, 23, 26 and 30 gauge needle 25, 25, 50, and 100 times respectively. 2  $\mu$ l samples of the sheared material is taken for fluorescence measurements after every 50 passes through the 30 gauge needle until the fluorescence units (FU) plateau (approx. 100-150x). Test compounds are prepared by dissolving an appropriate amount of test compound in 1x PBS to a final concentration of 1 mM (10x stock). If insoluble, the compound is dissolved in 1/10 volume of DMSO and diluted in 1x PBS to 1 mM. A further 1/10 dilution is also prepared to test each candidate at both 100  $\mu$ M and 10  $\mu$ M.

To perform the seeded extension assay, each sample is set up with 50  $\mu$ l of 200  $\mu$ M monomer, 125 FU sheared seed (a variable quantity dependent on the batch of seed, routinely 3-6  $\mu$ l) and 10  $\mu$ l of 10x modulator solution. The sample volume is then adjusted to a final volume of 100  $\mu$ l with 1x PBS. Two concentrations of each modulator typically are tested: 100  $\mu$ M and 10  $\mu$ M, equivalent to a 1:1 and a 1:10 molar ratio of monomer to modulator. The controls include an unseeded reaction to confirm that the fresh monomer contains no seed, and a seeded reaction in the absence of any modulators, as a reference to compare against candidate modulators. The assay is incubated at 37 °C for 6 h, taking 2  $\mu$ l samples hourly for fluorescence measurements. To measure fluorescence, a 2  $\mu$ l sample of A $\beta$  is added to 400  $\mu$ l of Thioflavin-T solution (50 mM Potassium Phosphate 10 mM Thioflavin-T pH 7.5). The samples are vortexed and the fluorescence is read in a 0.5 ml micro quartz cuvette at EX 450 nm and EM 482 nm (Hitachi 4500 Fluorimeter).

$\beta$ -amyloid aggregation results in enhanced emission of Thioflavin-T. Accordingly, samples including an effective inhibitory modulator compound exhibit reduced emission as compared to control samples without the modulator compound.

### **EXAMPLE 3:            Analysis of $\beta$ -Amyloid Modulator Compounds                                  Comprising D-Amino Acids**

In this example, D-amino acid-containing modulator compounds designed based upon the A $\beta$  aggregation core domain A $\beta$ <sub>17-21</sub> were prepared and tested for their ability to inhibit aggregation of natural  $\beta$ -amyloid peptide using aggregations assays as

- 47 -

described in Example 2. Abbreviations used in this example are: h- (free amino terminus), -oh (free carboxylic acid terminus), -nh<sub>2</sub> (amide terminus), CA (cholyl, the acyl portion of cholic acid), PEA (phenethylamide) and d (D-amino acid). Compounds in which the amino acid residues are in parentheses and preceded by "d" indicate that all amino acid residues are D-amino acids. For example, d(LVFFA) indicates D-Leu-D-Val-D-Phe-D-Phe-D-Ala.

The results from a first series of experiments, using N-terminally-cholyl modified compounds, are summarized below in Table I. The modulator compounds were evaluated in nucleation assays utilizing 5  $\mu$ M A $\beta$ <sub>1-40</sub> and either 2 or 5  $\mu$ M test compound (*i.e.*, 40 or 100 mole % inhibitor). The change in lag time ( $\Delta$ Lag) is presented as the ratio of the lag time observed in the presence of the test compound (at either 2 or 5  $\mu$ M) to the lag time of the control.

Table I

Ref. #	N-Term. Mod.	Peptide	C-Term. Mod.	$\Delta$ Lag	
				2 $\mu$ M	5 $\mu$ M
PPI-382	CA-	LVFFA (SEQ ID NO: 3)	-nh <sub>2</sub>	>10	>12
PPI-399	CA-	LVYFA (SEQ ID NO: 32)	-cooh	3.0	6
PPI-454	CA-	d(AFFVL)	-cooh	4.0	>12
PPI-457	CA-	d(LVFFA)	-nh <sub>2</sub>	ND*	>10
PPI-458	CA-	d(LVFF)	-nh <sub>2</sub>	>10	6

\* ND = not done

The results shown in Table I demonstrate that all D-amino acid-containing modulators designed based on the A $\beta$ <sub>17-21</sub> region are effective inhibitors of A $\beta$  aggregation. Effective inhibitors can comprise, for example, all D-amino acid compounds corresponding to the entire A $\beta$ <sub>17-21</sub> region (*e.g.*, PPI-457), to a smaller portion thereof (*e.g.*, PPI-458, comprising A $\beta$ <sub>17-20</sub>) or to a rearranged sequence thereof (*e.g.*, PPI-454). The carboxy terminus of effective inhibitors can comprise, for example, a free carboxylic acid terminus (*e.g.*, PPI-454) or a C-terminal amide modification (*e.g.*, PPI-457 and PPI-458).

25

In a second series of experiments using all D-amino acid modulators, a different stock of A $\beta$ <sub>1-40</sub> was used in the nucleation assays from that used for the experiments shown in Table I. This new stock exhibited some delay in lag time even in absence of

- 48 -

inhibitor and therefore the fold increase in lag time in the presence of test inhibitors was lower in these experiments compared to previous experiments. Despite this difference, the ability of a variety of all D-amino acid-containing modulators to inhibit A $\beta$  aggregation was evident compared to the negative control, an all D-alanine containing peptide (PPI-473). The results of this series of experiments, in which test compounds were assayed at 2, 3, 4 or 5  $\mu$ M, are shown below in Table II.

Table II

Ref. #	N-Term.	Peptide	C-Term.	$\Delta$ Lag			
	Mod.		Mod.	2 $\mu$ M	3 $\mu$ M	4 $\mu$ M	5 $\mu$ M
PPI-473	h-	d(AAAAAA)	-nh <sub>2</sub>	ND*	1.0	1.0	1.0
PPI-368	CA-	LVFFA (SEQ ID NO: 3)	-oh	ND	2	>3.5	3.5
				1.1	ND	ND	2.7
				ND	1.4	1.9	2.1
				ND	ND	2.1	>2.1
				ND	1.2	1.8	>2.5
PPI-455	CA-	d(LVYFA)	-oh	1.8	2.0	2.3	>2.7
PPI-458	CA-	d(LVFF)	-nh <sub>2</sub>	2.1	2.5	2.1	1.1
PPI-462	CA-	d(LV(IY)FA)	-oh	2.7	>2.7	>2.7	>2.7
PPI-463	CA-	d(LVF(IY)A)	-oh	>2.7	>2.7	>2.7	>2.7
PPI-464	h-	d(LVFFA)	-oh	1.3	1.9	>2.1	1.4
PPI-465	h-	d(LVFFA)	-nh <sub>2</sub>	1.9	3.5	>3.5	2.6
PPI-467	h-	d(VFF)	-nh <sub>2</sub>	1.2	1.4	1.0	1.1
PPI-471	h-	d(LVFF)	-nh <sub>2</sub>	2.6	2.6	2.6	2.4
PPI-479	h-	d(LVFA)	-oh	1.1	0.9	1.0	1.1
PPI-493	h-	d(VF)	-PEA	1.0	1.0	1.0	1.9
PPI-494	h-	d(LVF)	-PEA	1.0	1.3	1.4	1.4

\* ND = not done

10

The results shown in Table II further demonstrate that all D-amino acid-containing modulators designed based on the A $\beta$ <sub>17-21</sub> region are effective inhibitors of A $\beta$  aggregation.

#### 15 **EXAMPLE 4:** Variation of the N-Terminal Modifying Group on D-Amino Acid-Based Modulator Compounds

In this example, a series of modulator compounds were prepared which differed in their N-terminal modifying groups. The ability of the modulator compounds to inhibit aggregation of natural  $\beta$ -amyloid peptide was evaluated using aggregations assays as described in Example 2. Abbreviations used in this example and presentation

20

- 49 -

of the data are the same as described in Example 3. The results for compounds modified with N-terminal modifying groups derived from different bile acids are shown below in Table III. The results for compounds modified with various hydrophobic N-terminal modifying groups are shown below in Table IV. The results for compounds modified with various hydrophobic N-terminal modifying groups are shown below in Table IV. The results for compounds modified with various N-terminal hydroxylated and oxygenated modifying groups are shown below in Table V. Compounds exhibiting a change in lag time ( $\Delta\text{Lag}$ ) of 1.3 or greater are highlighted in bold.

10 Table III: Modifying Groups Derived from Bile Acids

Ref. #	N-Term. Mod.	Peptide	C-Term. Mod.	$\Delta\text{Lag}$ 5 $\mu\text{M}$
PPI-424	Cholyl-	LVFFA (SEQ ID NO: 3)	-oh	>6.0
PPI-425	Lithocholyl-	LVFFA (SEQ ID NO: 3)	-oh	1.4
PPI-520	Hyodeoxycholyl-	LVFFA (SEQ ID NO: 3)	-oh	>2.3
PPI-521	Chenodeoxycholyl-	LVFFA (SEQ ID NO: 3)	-oh	>2.3
PPI-522	Ursodeoxycholyl-	LVFFA (SEQ ID NO: 3)	-oh	>2.3

Table IV: Hydrophobic Modifying Groups

Ref. #	N-Term. Mod.	Peptide	C-Term. Mod.	$\Delta\text{Lag}$ 5 $\mu\text{M}$
PPI-480	Phenylacetyl-	d(LVFF)	-nh <sub>2</sub>	1.0
PPI-484	Diphenylacetyl-	d(LVFF)	-nh <sub>2</sub>	1.3
PPI-485	Triphenylacetyl-	d(LVFF)	-nh <sub>2</sub>	2.7
PPI-490	<i>trans</i> -Cinnamoyl-	d(LVFF)	-nh <sub>2</sub>	1.0
PPI-525	Butanoyl-	d(LVFFA)	-nh <sub>2</sub>	1.0
PPI-526	Isobutanoyl-	d(LVFFA)	-nh <sub>2</sub>	1.8
PPI-524	4-Methylvaleryl-	d(LVFFA)	-nh <sub>2</sub>	1.6
PPI-492	1-Adamantanecarbonyl-	d(LVFF)	-nh <sub>2</sub>	1.1
PPI-497	h-	d(VF)	-PEA	1.0
PPI-495	Acetyl-	d(VF)	-PEA	1.0
PPI-494	Cholyl-	d(LVF)	-PEA	1.5
PPI-467	h-	d(VFF)	-nh <sub>2</sub>	1.0
PPI-502	h-	d(VFF)	-oh	1.0

Table V: Hydroxylated and Oxygenated Modifying Groups

Ref. #	N-Term. Mod.	Peptide	C-Term. Mod.	$\Delta$ Lag
				5 $\mu$ M
PPI-483	3-Hydroxyphenylacetyl-	d(LVFF)	-nh <sub>2</sub>	2.2
PPI-487	3,5-Dihydroxy-2-naphthoyl-	d(LVFF)	-nh <sub>2</sub>	2.2
PPI-523	3,5-Dihydroxy-2-naphthoyl-	d(LVFFA)	-nh <sub>2</sub>	3.8
PPI-481	2-Hydroxyphenylacetyl-	d(LVFF)	-nh <sub>2</sub>	2.0
PPI-486	2,4-Dihydroxybenzoyl-	d(LVFF)	-nh <sub>2</sub>	1.2
PPI-489	Benzoylpropanoyl-	d(LVFF)	-nh <sub>2</sub>	1.2
PPI-491	3,4-Dihydroxycinnamoyl-	d(LVFF)	-nh <sub>2</sub>	3.0
PPI-482 (isomer 1)	( $\pm$ )-Mandelyl-	d(LVFF)	-nh <sub>2</sub>	1.5
PPI-482 (isomer 2)	( $\pm$ )-Mandelyl-	d(LVFF)	-nh <sub>2</sub>	1.6
PPI-518	( $\pm$ )-Mandelyl-( $\pm$ )-mandelyl-	d(LVFFA)	-nh <sub>2</sub>	1.5
PPI-516		Pepstatin A		1.6
PPI-537	4-Hydroxycinnamoyl-	d(LVFFA)	-nh <sub>2</sub>	1.0
PPI-535	Glycolyl-	d(LVFFA)	-nh <sub>2</sub>	1.4
PPI-596	Glycolyl-	d(FFFVL)	-nh <sub>2</sub>	3.6

The results shown in Tables III, IV and V demonstrate that a variety of different N-terminal modifying groups can be used in the inhibitory compounds of the invention.

5

**EXAMPLE 5: D-Amino Acid-Based Modulator Compounds Having a Free Amino-Terminus**

In this example, the necessity for an N-terminal modifying group on the D-amino acid-based modulator compounds was evaluated. Peptides comprised entirely of D-amino acids and having a free amino terminus were prepared and tested for their ability to inhibit aggregation of natural  $\beta$ -amyloid peptide using aggregations assays as described in Example 2. Abbreviations used in this example and presentation of the data are the same as described in Example 3. The results are shown below in Table VI.

Compounds exhibiting a change in lag time ( $\Delta$ Lag) of 1.3 or greater are highlighted in bold.

- 51 -

Table VI

Ref. #	N-Term. Mod.	Peptide	C-Term. Mod.	$\Delta$ Lag		
				0.5 $\mu$ M	1.5 $\mu$ M	5.0 $\mu$ M
PPI-500	h-	d(AFFVL)	-nh <sub>2</sub>	1.0	1.8	>6.0
PPI-503	h	d(LVYFA)	-nh <sub>2</sub>	ND	ND	1.2
PPI-504	h	d(LV(IodoY)FA)	-nh <sub>2</sub>	ND	ND	2.2
PPI-505	Acetyl-	d(LVYFA)	-nh <sub>2</sub>	ND	ND	1.0
PPI-506	Acetyl-	d(LV(IodoY)FA)	-nh <sub>2</sub>	ND	ND	1.0
PPI-577	h-	d(AFFLL)	-nh <sub>2</sub>	1.0	1.3	>7.5
PPI-578	h-	d(LFFVL)	-nh <sub>2</sub>	2.5	4.8	>7.5
PPI-579	h-	d(FFFVL)	-nh <sub>2</sub>	1.8	6.3	>7.5
PPI-533	h-	d(FFFLV)	-nh <sub>2</sub>	1.5	3.8	>7.5
PPI-589	h-	d(FFFFL)	-nh <sub>2</sub>	1.5	3.3	>7.5
PPI-598	h-	d(AFFFL)	-nh <sub>2</sub>	1.3	2.0	6.3

\* ND = not done

The results shown in Table VI demonstrate that modulators comprising all D-amino acids and having a free amino terminus are effective at inhibiting aggregation of natural  $\beta$ -amyloid peptides (*i.e.*, an N-terminal modifying group is not required for the D-amino acid-containing modulators to effectively inhibit aggregation of natural  $\beta$ -amyloid peptides). A particularly preferred D-amino acid modulator compound having a free amino-terminus is PPI-579, the retro-inverso isomer of A $\beta$ <sub>17-21</sub> (A<sub>21</sub>→F) with a C-terminal amide.

**EXAMPLE 6:****Neurotoxicity Assay**

The neurotoxicity of natural  $\beta$ -amyloid peptide aggregates, in either the presence or absence of a  $\beta$ -amyloid modulator, can be tested in a cell-based assay using either a rat or human neuronally-derived cell line (PC-12 cells or NT-2 cells, respectively) and the viability indicator 3,(4,4-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT). (See *e.g.*, Shearman, M.S. *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:1470-1474; Hansen, M.B. *et al.* (1989) *J. Immun. Methods* 119:203-210 for a description of similar cell-based viability assays). PC-12 is a rat adrenal pheochromocytoma cell line and is available from the American Type Culture Collection, Rockville, MD (ATCC CRL 1721). MTT (commercially available from Sigma Chemical Co.) is a chromogenic substrate that is converted from yellow to blue in viable cells, which can be detected spectrophotometrically.

- 52 -

To test the neurotoxicity of natural  $\beta$ -amyloid peptides, stock solutions of fresh  $A\beta$  monomers and aged  $A\beta$  aggregates are first prepared.  $A\beta_{1-40}$  in 100% DMSO is prepared from lyophilized powder and immediately diluted in one half the final volume in  $H_2O$  and then one half the final volume in 2X PBS so that a final concentration of 200  $\mu M$  peptide, 4% DMSO is achieved. Peptide prepared in this way and tested immediately on cells is referred to as "fresh"  $A\beta$  monomer. To prepare "aged"  $A\beta$  aggregates, peptide solution is placed in a 1.5 ml Eppendorf tube and incubated at 37 °C for eight days to allow fibrils to form. Such "aged"  $A\beta$  peptide can be tested directly on cells or frozen at -80°C. The neurotoxicity of fresh monomers and aged aggregates are tested using PC12 and NT2 cells. PC12 cells are routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum, 5% fetal calf serum, 4mM glutamine, and 1% gentamycin. NT2 cells are routinely cultured in OPTI-MEM medium (GIBCO BRL CAT. #31985) supplemented with 10% fetal calf serum, 2 mM glutamine and 1% gentamycin. Cells are plated at 10-15,000 cells per well in 90  $\mu l$  of fresh medium in a 96 -well tissue culture plate 3-4 hours prior to treatment. The fresh or aged  $A\beta$  peptide solutions (10  $\mu L$ ) are then diluted 1:10 directly into tissue culture medium so that the final concentration is in the range of 1-10  $\mu M$  peptide. Cells are incubated in the presence of peptide without a change in media for 48 hours at 37°C. For the final three hours of exposure of the cells to the  $\beta$ -AP preparation, MTT is added to the media to a final concentration of 1 mg/ml and incubation is continued at 37 °C. Following the two hour incubation with MTT, the media is removed and the cells are lysed in 100  $\mu L$  isopropanol/0.4N HCl with agitation. An equal volume of PBS is added to each well and the plates are agitated for an additional 10 minutes. Absorbance of each well at 570 nm is measured using a microtiter plate reader to quantitate viable cell.

Using this assay, the neurotoxicity of aged (5 day or 8 day)  $A\beta_{1-40}$  aggregates alone, but not fresh  $A\beta_{1-40}$  monomers alone, was confirmed. Experiments demonstrated that incubating the neuronal cells with increasing amounts of fresh  $A\beta_{1-40}$  monomers was not significantly toxic to the cells whereas incubating the cells with increasing amounts of 5 day or 8 day  $A\beta_{1-40}$  aggregates led to increasing amount of neurotoxicity. The  $EC_{50}$  for toxicity of aged  $A\beta_{1-40}$  aggregates was 1-2  $\mu M$  for both the PC12 cells and the NT2 cells.

To determine the effect of a  $\beta$ -amyloid modulator compound on the neurotoxicity of  $A\beta_{1-40}$  aggregates, a modulator compound is preincubated with  $A\beta_{1-40}$  monomers under standard nucleation assay conditions as described in Example 2 and at particular time intervals post-incubation, aliquots of the  $\beta$ -AP/modulator solution are

- 53 -

removed and 1) the turbidity of the solution is assessed as a measure of aggregation and 2) the solution is applied to cultured neuronal cells for 48 hours at which time cell viability is assessed using MTT to determine the neurotoxicity of the solution. Additionally, the ability of  $\beta$ -amyloid modulator compounds to reduce the neurotoxicity of preformed  $A\beta_{1-40}$  aggregates can be assayed. In these experiments,  $A\beta_{1-40}$  aggregates are preformed by incubation of the monomers in the absence of any modulators. The modulator compound is then incubated with the preformed  $A\beta_{1-40}$  aggregates for 24 hours at 37 °C, after which time the  $\beta$ -AP/modulator solution is collected and its neurotoxicity evaluated as described above.

10

**EXAMPLE 7:**                      **Assay of Modulator Compound Stability in Cerebrospinal Fluid**

The stability of a modulator compound in cerebrospinal fluid (CSF) can be assayed in an *in vitro* assay as follows. A CSF solution is prepared containing 75% Rhesus monkey CSF (commercially available from Northern Biomedical Research), 23% sterile phosphate buffered saline and 2% dimethylsulfoxide (v/v) (Aldrich Chemical Co., Catalog No. 27,685-5). Test modulator compounds are added to the CSF solution to a final concentration of 40  $\mu$ M or 15  $\mu$ M. All sample handling is carried out in a laminar flow hood and test solutions are maintained at 37 °C during the assay. After 24 hours, enzymatic activity in the solutions is quenched by adding acetonitrile to produce a final concentration of 25% (v/v). Samples (at the 0 time point and the 24 hour time point) are analyzed at room temperature using reverse-phase HPLC. A microbore column is used to maximize sensitivity. The parameters for analytical HPLC are as follows:

25

**Solvent System**

A: 0.1% Trifluoroacetic acid (TFA) in water (v/v)

B: 0.085% TFA/Acetonitrile, 1% H<sub>2</sub>O (v/v)

30

**Injection and Gradient**

Inject: 100-250  $\mu$ L of test sample

Run: 10% for B for 5 min., then 10-70% B over 60 min.



- 54 -

Chromatographic analysis is performed using a Hewlett Packard 1090 series II HPLC. The column used for separation is a C4, 5  $\mu$ m, 1 x 250 mm (Vydac #214TP51). The flow rate is 50  $\mu$ L/min and the elution profile of the test compounds is monitored at 214, 230, 260 and 280 nm.

5       The above-described CSF stability assay was used to compare the CSF stability of a L-amino acid-based modulator compound (PPI-368, having the structure cholyl-Leu-Val-Phe-Phe-Ala-OH) with an analogous D-amino acid-based peptide acid (PPI-433, having the structure cholyl-D-Leu-D-Val-D-Phe-D-Phe-D-Ala-OH) and an analogous D-amino acid-based peptide amide (PPI-457, having the structure cholyl-D-  
10   Leu-D-Val-D-Phe-D-Phe-D-Ala-NH<sub>2</sub>). The results, summarized in the bar graph shown in Figure 1, demonstrate that both D-amino acid-based compounds exhibit significantly greater stability in CSF than the L-amino acid-based compound.

**EXAMPLE 8:****Brain Uptake Assay**

15

Brain uptake of test modulator compounds is measured using the technique of Oldendorf (*Brain Research* (1970) 24:372-376). In this established model, the brain uptake index (BUI) is an estimate of the relative ability of a particular compound to cross the blood-brain barrier, expressed as a percentage of that observed by the freely  
20   diffusable reference, water. Radiolabelled compounds are administered to a test animal as a rapid bolus (200  $\mu$ l) into the left common carotid artery (with the left external carotid artery ligated). The animal is sacrificed 15 seconds later and the amount of radioactivity within the ipsilateral forebrain is determined. The BUI is computed using the equation below:

25

$$\text{Brain Uptake Index (BUI)} = \frac{(\text{dpm of test compound in brain})/(\text{dpm of water in brain})}{(\text{dpm of test compound in injectate})/(\text{dpm of water in injectate})}$$

The above-described assay was used to measure the brain uptake of four cholyl-  
30   modified-modulator compounds: PPI-382 (having the structure cholyl-Leu-Val-Phe-Phe-Ala-NH<sub>2</sub>) (SEQ ID NO: 33), PPI-457 (having the structure cholyl-D-Leu-D-Val-D-Phe-D-Phe-D-Ala-NH<sub>2</sub>), PPI-458 (having the structure cholyl-D-Leu-D-Val-D-Phe-D-Phe-NH<sub>2</sub>) and PPI-494 (having the structure cholyl-D-Leu-D-Val-D-Phe-phenethylamide). Radiolabel was introduced into the test compounds by using <sup>14</sup>C-  
35   labelled cholic acid for modification. The vehicle used for the test compounds was 50

- 55 -

mM cyclodextrin in 75% phosphate buffered saline. Water was used as the freely diffusable reference, sucrose was used as a negative control and cholic acid was used as a control for the diffusability of the modifying group. The results are summarized below in Table VII.

5

Table VII.

<u>Compound</u>	<u>Brain Uptake Index (<math>\pm</math> SEM)</u>
Water	100
Sucrose	$0.78 \pm 0.05$
Cholic Acid	$1.02 \pm 0.09$
PPI-382	$1.79 \pm 0.04$
PPI-457	$3.09 \pm 0.34$
PPI-458	$4.25 \pm 0.49$
PPI-494	$4.78 \pm 0.36$

The results indicate that the D-amino acid-based compounds (PPI-457, PPI-458 and PPI-494) exhibited greater brain uptake than the L-amino acid-based compound (PPI-382).

- 10 An acetyl-modified D-amino acid based compound (PPI-472, having the structure acetyl-D-Leu-D-Val-D-Phe-D-Phe-NH<sub>2</sub>) exhibited a similar brain uptake index as PPI-458 and PPI-494 (*i.e.*, about 4.5).

**EXAMPLE 9:****Analysis of Additional Compounds**

15

Additional compounds were tested using the methods described above. The results are summarized in the following tables.

Table VIII

<u>Ref. #</u>	<u>N-Term. Mod.</u>	<u>Peptide</u>	<u>C-Term. Mod.</u>	<u><math>\Delta</math>Lag 5 <math>\mu</math>M</u>
PPI-523	3,5-Dihydroxy-2-Napthoyl-	d(LVFFA)	-nh <sub>2</sub>	2.1
PPI-548	3,7-Dihydroxy-2-napththoyl-	d(LVFFA)	-nh <sub>2</sub>	2.5
PPI-558	4-Hydroxybenzoyl-	d(LVFFA)	-nh <sub>2</sub>	2.0
PPI-559	4-Hydroxyphenylacetyl-	d(LVFFA)	-nh <sub>2</sub>	3.5

20

- 56 -

Table IX

Ref. #	N-Term. Mod.	Peptide	C-Term. Mod.	$\Delta$ Lag 5 $\mu$ M
PPI-457	Cholyl-	d(LVFFA)	-nh <sub>2</sub>	>6
PPI-483	3-Hydroxyphenylacetyl-	d(LVFF)	-nh <sub>2</sub>	2.9
PPI-494	Cholyl-	d(LVF)	Phenethyl- amide	1.3
PPI-501	Ac-	d(LVFFA)	-nh <sub>2</sub>	1.4
PPI-520	Hyodeoxycholyl-	(LVFFA)	-oh	1.5
PPI-521	Chenodeoxycholyl-	(LVFFA)	-oh	3.6
PPI-522	Ursodeoxycholyl-	(LVFFA)	-oh	1.2
PPI-532	2,4-Dihydroxybenzoyl-	d(LVFFA)	-nh <sub>2</sub>	1.7
PPI-535	Glycolyl-	d(LVFFA)	-nh <sub>2</sub>	1.3
PPI-536	2-Hydroxycinnamoyl-	d(LVFFA)	-nh <sub>2</sub>	1.4
PPI-537	4-Hydroxycinnamoyl-	d(LVFFA)	-nh <sub>2</sub>	1.6
PPI-538	3-Hydroxycinnamoyl-	d(LVFFA)	-nh <sub>2</sub>	2.0
PPI-539	5-Hydantoinacetyl-	d(LVFFA)	-nh <sub>2</sub>	2.4
PPI-543	3,4-Dihydroxycinnamoyl-	d(LVFFA)	-nh <sub>2</sub>	1.6
PPI-546	3-Formylbenzoyl-	d(LVFFA)	-nh <sub>2</sub>	1.9
PPI-554	2-Formylphenoxyacetyl-	d(LVFFA)	-nh <sub>2</sub>	2.6
PPI-555	4-Hydroxy-3-methoxycinnamoyl-	d(LVFFA)	-nh <sub>2</sub>	1.7
PPI-568	3,5-Dihydroxy-2-naphthoyl-	d(AFFVL)	-nh <sub>2</sub>	>6
PPI-569	3,4-Dihydroxycinnamoyl-	d(AFFVL)	-nh <sub>2</sub>	2.6
PPI-584	( $\pm$ )-Mandelyl-	d(AFFVL)	-nh <sub>2</sub>	4.8
PPI-587	4-Hydroxycinnamoyl-	d(AFFVL)	-nh <sub>2</sub>	3.7
PPI-592	Glycolyl-	d(AFFLL)	-nh <sub>2</sub>	1.8
PPI-593	3,4-Dihydroxycinnamoyl-	d(LFFVL)	-nh <sub>2</sub>	2.0
PPI-596	Glycolyl-	d(FFFVL)	-nh <sub>2</sub>	3.6
PPI-599	DL-3-(4-Hydroxyphenyl)Lactyl-	d(LVFFA)	-nh <sub>2</sub>	1.4
PPI-607	D-3-Phenyllactyl-	d(AFFVL)	-nh <sub>2</sub>	3.1
PPI-608	L-3-Phenyllactyl-	d(AFFVL)	-nh <sub>2</sub>	>6
PPI-610	3-(2-Hydroxyphenyl)propionyl-	d(AFFVL)	-nh <sub>2</sub>	4.5
PPI-611	4-(2-Hydroxyphenyl)propionyl-	d(AFFVL)	-nh <sub>2</sub>	3.2
PPI-612	D-3-Phenyllactyl-	d(FFVL)	-nh <sub>2</sub>	2.1
PPI-615	3-(2-Hydroxyphenyl)propionyl-	d(FFVL)	-nh <sub>2</sub>	2.0
PPI-616	3-(4-Hydroxyphenyl)propionyl-	d(FFVL)	-nh <sub>2</sub>	1.5
PPI-619	Hydrocinnamoyl-	d(FFVL)	-ch	2.0
PPI-629	4-Hydroxybenzoyl-	d(AFFVL)	-nh <sub>2</sub>	2.9
PPI-635	D-3-Phenyllactyl-	d(FFFVL)	-nh <sub>2</sub>	2.5
PPI-636	Hydrocinnamoyl-	d(FFFVL)	-nh <sub>2</sub>	1.7
PPI-643	4-Hydroxybenzoyl-	d(LFFFL)	-nh <sub>2</sub>	2.2
PPI-644	Ac-	d(LFFFL)	-nh <sub>2</sub>	2.1
PPI-648	3,7-Dihydroxy-2-naphthoyl-	d(LFFVL)	-nh <sub>2</sub>	3.1
PPI-649	4-Hydroxyphenylacetyl-	d(LFFVL)	-nh <sub>2</sub>	5.1

- 57 -

PPI-650	3,7-Dihydroxy-2-naphthoyl-	d(FFFVL)	-nh <sub>2</sub>	2.2
PPI-651	4-Hydroxyphenylacetyl-	d(FFFVL)	-nh <sub>2</sub>	2.1

Table X

<u>Ref. #</u>	<u>N-Term.</u> <u>Mod.</u>	<u>Peptide</u>	<u>C-Term.</u> <u>Mod.</u>	<u>ΔLag</u> <u>5 μM</u>
PPI-504	H-	d(LV[iodo-Y]FA)	-nh <sub>2</sub>	1.4
PPI-533	H-	d(FFFLV)	-nh <sub>2</sub>	>6
PPI-571	H-	d(A[homoPhe][homoPhe]VL)	-nh <sub>2</sub>	>6
PPI-577	H-	d(AFFLL)	-nh <sub>2</sub>	>6
PPI-578	H-	d(LFFVL)	-nh <sub>2</sub>	3.2
PPI-579	H-	d(FFFVL)	-nh <sub>2</sub>	>6
PPI-589	H-	d(FFFFL)	-nh <sub>2</sub>	>6
PPI-598	H-	d(AFFFL)	-nh <sub>2</sub>	>6
PPI-602	H-	d(FFVL)	-nh <sub>2</sub>	1.6
PPI-638	H-	d(LFFFL)	-nh <sub>2</sub>	>6
PPI-655	H-	d(LVFFL)	-nh <sub>2</sub>	>6

5

Table XI

<u>Ref. #</u>	<u>N-Term Mod.</u>	<u>Peptide</u>	<u>C-Term</u> <u>Mod.</u>	<u>μM</u>	<u>ΔLag</u>	<u>% Inhib.</u> <u>Plateau</u>
PPI-656	H-	d(LVFFF)	-nh <sub>2</sub>	5	>2.5	60
				1.5	6	60
				0.5	2.3	0
PPI-657	H-	d(LVFFV)	-nh <sub>2</sub>	5	>10	100
				1.5	4	42
				0.5	1.7	0
PPI-664	3,7-Dihydroxy-2-naphthol-	d(Pal-FFVL)	-nh <sub>2</sub>	5	2	28
				1.5	0	19
				0.5	0	0
PPI-665	4-Hydroxybenzoyl-	d(Pal-FFVL)	-nh <sub>2</sub>	5	2	0
				1.5	2	0
				0.5	1.5	0
PPI-730	3-Pyridylacetyl-	d(AFFVL)	-nh <sub>2</sub>	5	2	50
PPI-732	Isonicotinoyl-	d(AFFVL)	-nh <sub>2</sub>	5	<2	0
PPI-733	H-Glycyl-	d(FFVL)	-nh <sub>2</sub>	5	0	25
PPI-734	H-Glycyl-	d(AFFVL)	-nh <sub>2</sub>	5	>10	100
PPI-741	4-Pyridylacetyl-	d(AFFVL)	-nh <sub>2</sub>	5	ND	0
				2.5	2.0	ND
PPI-746	4-Quinolinecarboxyl-	d(AFFVL)	-nh <sub>2</sub>	5	2	0
PPI-748	1-Isoquinolinecarboxyl-	d(AFFVL)	-nh <sub>2</sub>	5	<3	0
PPI-749	3-Isoquinolinecarboxyl-	d(AFFVL))	-nh <sub>2</sub>	5	3	28

- 58 -

PPI-772	4-Hydroxybenzoyl-	d(LVFYA)	-nh <sub>2</sub>	5	2	0
PPI-774	4-Hydroxybenzoyl-	d(LVYFA)	-nh <sub>2</sub>	5	1	0
PPI-775	4-Hydroxybenzoyl-	d(LVYYA)	-nh <sub>2</sub>	5	1	0

Pal = pyridylalaline

ND=not done

**EXAMPLE 10:****Brain Uptake of PPI-558**

5

A brain uptake assay was used to measure the brain uptake of tritium-labelled PPI-558 (<sup>3</sup>H-PPI-558) (having the structure: 4-Hydroxybenzoyl-D-Leu-D-Val-D-Phe-D-Phe-D-Ala-NH<sub>2</sub>). The tritium-labelled PPI-558 was prepared via the synthesis of the iodo-phenylalanine analog of PPI-558, followed by reductive tritiation of the label and HPLC purification. Such labeling can be performed as a commercial service by, for example, Amersham or New England Nuclear.

Male Sprague-Dawley rats (259-280 g) received a single subcutaneous injection in the nape of the neck (4.6 mg/kg at 1 ml/kg = 4.6 mg/ml in 100% Sesame Oil). At various time points post administration (2, 8 and 24 hours; n=4/time point), the animals were anesthetized. Trunk blood was taken to determine plasma levels of the parent compound and the left side of the brain was perfused with 1 ml of saline over 30 seconds via the left common carotid artery. The brain was rapidly removed and the left forebrain (perfused) was subjected to capillary depletion. This technique, plus perfusion, removes blood and brain capillaries from the parenchyma and thus allows the accurate determination of the levels of PPI-558 that have traversed the blood brain barrier into the parenchyma.

The concentration of the parent compound (<sup>3</sup>H-PPI-558) (mean ± sem) was determined in the plasma (results shown in the graph of Figure 2) and in the brain parenchyma (results shown in the graph of Figure 3).

As highlighted in Figure 4 (which shows the ratio of brain versus plasma levels of PPI-558), the data shows that following a single subcutaneous injection of 4.6 mg/kg of PPI-558, there was 7.4 nM in the plasma at 2 hours with brain parenchymal levels of almost twice that (14.1 nM). Similar profiles were seen at 8 and 24 hours post administration.

The data confirms the possibility that brain clearance is slower than that at plasma (seen in i.v. bolus studies) and that by maintaining plasma levels, brain levels can be maintained.

- 59 -

**EXAMPLE 11: Safety Profile for PPI-558**

PPI-558 at 3 and 30 mg/kg (in 100% Sesame Oil) was administered to female Sprague-Dawley rats as a single subcutaneous injection each day for 14 days. On day 15, animals were sacrificed 1 hour post administration. Via Mass Spectroscopy plasma levels of  $4.9 \pm 1.1$  ng/ml and  $21.8 \pm 2.0$  ng/ml were observed for the 3 and 30 mg/kg groups, respectively. No overt toxicity was observed. Blood chemistry, hematology was within normal ranges and histological analysis of various organs revealed no problems.

10

Forming part of this disclosure is the appended Sequence Listing, the contents of which are summarize in the Table below.

SEQ ID NO:	A $\beta$ Amino Acids	Peptide Sequence
1	43 amino acids	A $\beta$ <sub>1-43</sub>
2	103 amino acids	APP C-terminus
3	A $\beta$ <sub>17-21</sub>	LVFFA
4	A $\beta$ <sub>17-21</sub> retro-inverso isomer	d(AFFVL)
5	A $\beta$ <sub>17-21</sub> (V <sub>18</sub> →L) retro-inverso isomer	d(AFFLL)
6	A $\beta$ <sub>17-21</sub> (A <sub>21</sub> →L) retro-inverso isomer	d(LFFVL)
7	A $\beta$ <sub>17-21</sub> (A <sub>21</sub> →F) retro-inverso isomer	d(FFFVL)
8	A $\beta$ <sub>17-20</sub>	LVFF
9	A $\beta$ <sub>17-20</sub> inverso isomer	d(LVFF)
10	A $\beta$ <sub>17-19</sub> inverso isomer (C-term. mod.)	d(LVF)phenethylamide
11	A $\beta$ <sub>17-20</sub> (F <sub>19</sub> →Y) inverso isomer	d(LVYF)
12	A $\beta$ <sub>17-20</sub> (F <sub>19</sub> →IodoY) inverso isomer	d(LV(IodoY)F)
13	A $\beta$ <sub>17-20</sub> (F <sub>20</sub> →Y) inverso isomer	d(LVFY)
14	A $\beta$ <sub>17-20</sub> (F <sub>20</sub> →IodoY) inverso isomer	d(LVF(IodoY))
15	A $\beta$ <sub>17-20</sub> (F <sub>20</sub> →A) inverso isomer	d(LVFA)
16	A $\beta$ <sub>17-21</sub> inverso isomer	d(LVFFA)
17	A $\beta$ <sub>17-21</sub> (L <sub>17</sub> →A; A <sub>21</sub> →L) inverso isomer	d(AVFFL)
18	A $\beta$ <sub>17-21</sub> (F <sub>19</sub> →Y) inverso isomer	d(LVYFA)
19	A $\beta$ <sub>17-21</sub> (F <sub>19</sub> →IodoY) inverso isomer	d(LV(IodoY)FA)
20	A $\beta$ <sub>17-21</sub> (F <sub>20</sub> →Y) inverso isomer	d(LVFYA)
21	A $\beta$ <sub>17-21</sub> (F <sub>20</sub> →IodoY) inverso isomer	d(LVF(IodoY)A)

- 60 -

22	A $\beta$ <sub>17-20</sub> retro-inverso isomer	d(FFVL)
23	A $\beta$ <sub>18-21</sub> retro-inverso isomer	d(AFFV)
24	A $\beta$ <sub>17-21</sub> (L <sub>17</sub> →V; V <sub>18</sub> →L; A <sub>21</sub> →F) retro-inverso isomer	d(FFFLV)
25	A $\beta$ <sub>17-21</sub> (V <sub>18</sub> →F; A <sub>21</sub> →F) retro-inverso isomer	d(FFFFL)
26	A $\beta$ <sub>17-21</sub> (V <sub>18</sub> →F) retro-inverso isomer	d(AFFFL)
27	A $\beta$ <sub>17-21</sub> (A <sub>21</sub> →F)	d(LVFFF)
28	A $\beta$ <sub>17-21</sub> (A <sub>21</sub> →V)	d(LVFFV)
29	A $\beta$ <sub>17-21</sub> (F <sub>19</sub> →Y, F <sub>20</sub> →Y)	d(LVYYA)
30	A $\beta$ <sub>17-21</sub> (V <sub>18</sub> →F, A <sub>21</sub> →L)	d(LFFFL)
31	N/A	DDIIL-Adp
32	A $\beta$ <sub>17-21</sub> (F <sub>19</sub> →Y)	LVYFA
33	A $\beta$ <sub>17-21</sub>	cholyL-LVFFA-amide

### EQUIVALENTS

- Those skilled in the art will recognize, or be able to ascertain using no more than  
5 routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

- 61 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANT:  
(A) NAME: PRAECIS PHARMACEUTICALS INCORPORATED  
(B) STREET: ONE HAMPSHIRE STREET  
(C) CITY: CAMBRIDGE  
(D) STATE: MASSACHUSETTS  
10 (E) COUNTRY: USA  
(F) POSTAL CODE (ZIP): 02139-1572  
(G) TELEPHONE: (617) 494-8400  
(H) TELEFAX: (617) 494-8414
- 15 (ii) TITLE OF INVENTION: Modulators of  $\beta$ -Amyloid Peptide  
Aggregation Comprising D-Amino Acids
- (iii) NUMBER OF SEQUENCES: 33
- 20 (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: LAHIVE & COCKFIELD  
(B) STREET: 28 State Street  
(C) CITY: Boston  
(D) STATE: Massachusetts  
25 (E) COUNTRY: USA  
(F) ZIP: 02109-1875
- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy disk  
30 (B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:  
35 (A) APPLICATION NUMBER:  
(B) FILING DATE: Herewith  
(C) CLASSIFICATION:
- (ix) PRIOR APPLICATION DATA:  
40 (A) APPLICATION NUMBER: USSN 08/703,675  
(B) FILING DATE: 27-AUG-1996
- (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER:  
45 (B) FILING DATE: 21-JUL-1997
- (x) ATTORNEY/AGENT INFORMATION:  
(A) NAME: KARA, Catherine J.  
(B) REGISTRATION NUMBER: 41,106  
50 (C) REFERENCE/DOCKET NUMBER: PPI-016CP2PC
- (xi) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: (617) 227-7400



- 62 -

(B) TELEFAX: (617)227-5941

## (2) INFORMATION FOR SEQ ID NO:1:

5

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 43 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asp	Ala	Glu	Phe	Arg	His	Asp	Ser	Gly	Tyr	Glu	Val	His	His	Gln	Lys
1				5					10					15	
Leu	Val	Phe	Phe	Ala	Glu	Asp	Val	Gly	Ser	Asn	Lys	Gly	Ala	Ile	Ile
			20					25					30		
Gly	Leu	Met	Val	Gly	Gly	Val	Val	Ile	Ala	Thr					
		35					40								

25

## (2) INFORMATION FOR SEQ ID NO:2:

30

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 103 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

40

Glu	Val	Lys	Met	Asp	Ala	Glu	Phe	Arg	His	Asp	Ser	Gly	Tyr	Glu	Val
1				5					10					15	
His	His	Gln	Lys	Leu	Val	Phe	Phe	Ala	Glu	Asp	Val	Gly	Ser	Asn	Lys
			20					25					30		
Gly	Ala	Ile	Ile	Gly	Leu	Met	Val	Gly	Gly	Val	Val	Ile	Ala	Thr	Val
			35				40					45			
Ile	Val	Ile	Thr	Leu	Val	Met	Leu	Lys	Lys	Lys	Gln	Tyr	Thr	Ser	Ile
	50					55					60				
His	His	Gly	Val	Val	Glu	Val	Asp	Ala	Ala	Val	Thr	Pro	Glu	Glu	Arg
65					70					75				80	

50

- 63 -

His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys  
85 90 95

5 Phe Phe Glu Gln Met Gln Asn  
100

(2) INFORMATION FOR SEQ ID NO:3:

10

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

20 Leu Val Phe Phe Ala  
5

(2) INFORMATION FOR SEQ ID NO:4:

25

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified site  
(B) LOCATION: 1-5  
(D) OTHER INFORMATION: /note= D amino acid

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

40 Ala Phe Phe Val Leu  
5

(2) INFORMATION FOR SEQ ID NO:5:

45

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- 64 -

- (A) NAME/KEY: Modified site
- (B) LOCATION: 1-5
- (D) OTHER INFORMATION: /note= D amino acid

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ala Phe Phe Leu Leu  
5

10

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- 15 (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

20

(ix) FEATURE:

- (A) NAME/KEY: Modified site
- (B) LOCATION: 1-5
- (D) OTHER INFORMATION: /note= D amino acid

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Leu Phe Phe Val Leu  
5

30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5 amino acids
- 35 (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

40

(ix) FEATURE:

- (A) NAME/KEY: Modified site
- (B) LOCATION: 1-5
- (D) OTHER INFORMATION: /note= D amino acid

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Phe Phe Phe Val Leu  
5

50

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- 65 -

(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Leu Val Phe Phe

10

(2) INFORMATION FOR SEQ ID NO:9:

15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified site  
(B) LOCATION: 1-4

25 (D) OTHER INFORMATION: /note= D amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Leu Val Phe Phe

30

(2) INFORMATION FOR SEQ ID NO:10:

35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 3 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified site  
(B) LOCATION: 1-3

45 (D) OTHER INFORMATION: /note= D amino acid

(ix) FEATURE:

(A) NAME/KEY: Modified site  
(B) LOCATION: 3

50 (D) OTHER INFORMATION: /note= phenethylamide C-terminal  
modification

- 66 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Leu Val Phe

5

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

15

(ix) FEATURE:

- (A) NAME/KEY: Modified site  
(B) LOCATION: 1-4  
(D) OTHER INFORMATION: /note= D amino acid

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Leu Val Tyr Phe

25

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

35

(ix) FEATURE:

- (A) NAME/KEY: Modified site  
(B) LOCATION: 1-4  
(D) OTHER INFORMATION: /note= D amino acid

40

(ix) FEATURE:

- (A) NAME/KEY: Modified site  
(B) LOCATION: 3  
(D) OTHER INFORMATION: /note= Xaa=iodotyrosine

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Leu Val Xaa Phe

50

(2) INFORMATION FOR SEQ ID NO:13:

- 67 -

(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 4 amino acids  
    (B) TYPE: amino acid  
5    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:  
10    (A) NAME/KEY: Modified site  
    (B) LOCATION: 1-4  
    (D) OTHER INFORMATION: /note= D amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:  
15    Leu Val Phe Tyr

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 4 amino acids  
    (B) TYPE: amino acid  
25    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:  
30    (A) NAME/KEY: Modified site  
    (B) LOCATION: 1-4  
    (D) OTHER INFORMATION: /note= D amino acid

(ix) FEATURE:  
35    (A) NAME/KEY: Modified site  
    (B) LOCATION: 4  
    (D) OTHER INFORMATION: /note= Xaa=iodotyrosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:  
40    Leu Val Phe Xaa

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 4 amino acids  
    (B) TYPE: amino acid  
50    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

- 68 -

(ix) FEATURE:  
    (A) NAME/KEY: Modified site  
    (B) LOCATION: 1-4  
    (D) OTHER INFORMATION: /note= D amino acid  
5  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:  
    Leu Val Phe Ala  
10  
(2) INFORMATION FOR SEQ ID NO:16:  
    (i) SEQUENCE CHARACTERISTICS:  
15        (A) LENGTH: 5 amino acids  
        (B) TYPE: amino acid  
        (D) TOPOLOGY: linear  
    (ii) MOLECULE TYPE: peptide  
20  
    (ix) FEATURE:  
        (A) NAME/KEY: Modified site  
        (B) LOCATION: 1-5  
        (D) OTHER INFORMATION: /note= D amino acid  
25  
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:  
        Leu Val Phe Phe Ala  
                            5  
30  
(2) INFORMATION FOR SEQ ID NO:17:  
    (i) SEQUENCE CHARACTERISTICS:  
35        (A) LENGTH: 5 amino acids  
        (B) TYPE: amino acid  
        (D) TOPOLOGY: linear  
    (ii) MOLECULE TYPE: peptide  
40  
    (ix) FEATURE:  
        (A) NAME/KEY: Modified site  
        (B) LOCATION: 1-5  
        (D) OTHER INFORMATION: /note= D amino acid  
45  
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:  
        Ala Val Phe Phe Leu  
                            5  
50  
(2) INFORMATION FOR SEQ ID NO:18:

- 69 -

(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 5 amino acids  
    (B) TYPE: amino acid  
    (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:  
    (A) NAME/KEY: Modified site  
10      (B) LOCATION: 1-5  
    (D) OTHER INFORMATION: /note= D amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

15      Leu Val Tyr Phe Ala  
                            5

(2) INFORMATION FOR SEQ ID NO:19:

20

(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 5 amino acids  
    (B) TYPE: amino acid  
    (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:  
    (A) NAME/KEY: Modified site  
30      (B) LOCATION: 1-5  
    (D) OTHER INFORMATION: /note= D amino acid

(ix) FEATURE:  
    (A) NAME/KEY: Modified site  
35      (B) LOCATION: 3  
    (D) OTHER INFORMATION: /note= Xaa=iodotyrosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

40      Leu Val Xaa Phe Ala  
                            5

(2) INFORMATION FOR SEQ ID NO:20:

45

(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 5 amino acids  
    (B) TYPE: amino acid  
    (D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:



- 70 -

- (A) NAME/KEY: Modified site
  - (B) LOCATION: 1-5
  - (D) OTHER INFORMATION: /note= D amino acid
- 5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:  

Leu Val Phe Tyr Ala  
5
- 10 (2) INFORMATION FOR SEQ ID NO:21:
  - (i) SEQUENCE CHARACTERISTICS:
    - 15 (A) LENGTH: 5 amino acids
    - (B) TYPE: amino acid
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
- 20 (ix) FEATURE:
  - (A) NAME/KEY: Modified site
  - (B) LOCATION: 1-5
  - (D) OTHER INFORMATION: /note= D amino acid
- 25 (ix) FEATURE:
  - (A) NAME/KEY: Modified site
  - (B) LOCATION: 4
  - (D) OTHER INFORMATION: /note= Xaa=iodotyrosine
- 30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:  

Leu Val Phe Xaa Ala  
5
- 35 (2) INFORMATION FOR SEQ ID NO:22:
  - (i) SEQUENCE CHARACTERISTICS:
    - 40 (A) LENGTH: 4 amino acids
    - (B) TYPE: amino acid
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: peptide
- 45 (ix) FEATURE:
  - (A) NAME/KEY: Modified site
  - (B) LOCATION: 1-4
  - (D) OTHER INFORMATION: /note= D amino acid
- 50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:  

Phe Phe Val Leu

- 71 -

(2) INFORMATION FOR SEQ ID NO:23:

5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified site  
(B) LOCATION: 1-4

15 (D) OTHER INFORMATION: /note= D amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Ala Phe Phe Val

20

(2) INFORMATION FOR SEQ ID NO:24:

25 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

```

30      (ii) MOLECULE TYPE: peptide

```

(ix) FEATURE:

(A) NAME/KEY: Modified site  
(B) LOCATION: 1-5

35 (D) OTHER INFORMATION: /note= D amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Phe Phe Phe Leu Val

40 5

(2) INFORMATION FOR SEQ ID NO:25:

45 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified site

- 72 -

(B) LOCATION: 1-5

(D) OTHER INFORMATION: /note= D amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

5

Phe Phe Phe Phe Leu

5

10 (2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

15

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

20

(A) NAME/KEY: Modified site

(B) LOCATION: 1-5

(D) OTHER INFORMATION: /note= D amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

25

Ala Phe Phe Phe Leu

5

(2) INFORMATION FOR SEQ ID NO:27:

30

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

40

(A) NAME/KEY: Modified site

(B) LOCATION: 1-5

(D) OTHER INFORMATION: /note= D amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

45

Leu Val Phe Phe Phe

5

(2) INFORMATION FOR SEQ ID NO:28:

50

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

- 73 -

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

5 (A) NAME/KEY: Modified site  
(B) LOCATION: 1-5  
(D) OTHER INFORMATION: /note= D amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

10 Leu Val Phe Phe Val  
5

(2) INFORMATION FOR SEQ ID NO:29:

15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: peptide

(ix) FEATURE:

25 (A) NAME/KEY: Modified site  
(B) LOCATION: 1-5  
(D) OTHER INFORMATION: /note= D amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

30 Leu Val Tyr Tyr Ala  
5

(2) INFORMATION FOR SEQ ID NO:30:

35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 5 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: peptide

(ix) FEATURE:

45 (A) NAME/KEY: Modified site  
(B) LOCATION: 1-5  
(D) OTHER INFORMATION: /note= D amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

50 Leu Phe Phe Phe Leu  
5

(2) INFORMATION FOR SEQ ID NO:31:

- 74 -

- 5 (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 5 amino acids  
    (B) TYPE: amino acid  
    (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- 10 (ix) FEATURE:  
    (A) NAME/KEY: Modified site  
    (B) LOCATION: 5  
    (D) OTHER INFORMATION: /note= Adp modification
- 15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:  
    Asp Asp Ile Ile Leu  
        5
- 20 (2) INFORMATION FOR SEQ ID NO:32:
- 25 (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 5 amino acids  
    (B) TYPE: amino acid  
    (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- 30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:  
    Leu Val Tyr Phe Ala  
        5
- 35 (2) INFORMATION FOR SEQ ID NO:33:
- 40 (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 5 amino acids  
    (B) TYPE: amino acid  
    (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- 45 (ix) FEATURE:  
    (A) NAME/KEY: Modified site  
    (B) LOCATION: 1  
    (D) OTHER INFORMATION: /note= cholyl modification
- 50 (ix) FEATURE:  
    (A) NAME/KEY: Modified site  
    (B) LOCATION: 5  
    (D) OTHER INFORMATION: /note= amide modification

- 75 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Leu Val Phe Phe Ala

5

5

- 76 -

We claim:

1. A compound having the structure:

5

A-(Xaa)-B

wherein (Xaa) is a peptidic structure selected from the group consisting of D-Leu-D-Val-D-Phe-D-Phe, D-Leu-D-Val-D-Phe-phenethylamide, D-Leu-D-Val-D-Tyr-D-Phe, D-Leu-D-Val-D-IodoTyr-D-Phe, D-Leu-D-Val-D-Phe-D-Tyr, D-Leu-D-Val-D-Phe-D-IodoTyr, D-Leu-D-Val-D-Phe-D-Ala, D-Leu-D-Val-D-Phe-D-Phe-D-Ala, D-Ala-D-Val-D-Phe-D-Phe-D-Leu, D-Leu-D-Val-D-Tyr-D-Phe-D-Ala, D-Leu-D-Val-D-IodoTyr-D-Phe-D-Ala, D-Leu-D-Val-D-Phe-D-Tyr-D-Ala, D-Leu-D-Val-D-Phe-D-IodoTyr-D-Ala, D-Phe-D-Phe-D-Val-D-Leu, D-Ala-D-Phe-D-Phe-D-Val, D-Ala-D-Phe-D-Phe-D-Val-D-Leu, D-Ala-D-Phe-D-Phe-D-Leu-D-Leu, D-Leu-D-Phe-D-Phe-D-Val-D-Leu, D-Phe-D-Phe-D-Phe-D-Val-D-Leu, D-Phe-D-Phe-D-Phe-D-Leu-D-Val, D-Phe-D-Phe-D-Phe-D-Phe-D-Leu and D-Ala-D-Phe-D-Phe-D-Phe-D-Leu.

A is an amino-terminal modifying group selected from the group consisting of phenylacetyl, diphenylacetyl, triphenylacetyl, butanoyl, isobutanoyl hexanoyl, propionyl, 3-hydroxybutanoyl, 4-hydroxybutanoyl, 3-hydroxypropionyl, 2, 4-dihydroxybutyryl, 1-Adamantanecarbonyl, 4-methylvaleryl, 2-hydroxyphenylacetyl, 3-hydroxyphenylacetyl, 4-hydroxyphenylacetyl, 3,5-dihydroxy-2-naphthoyl, 3,7-dihydroxy-2-naphthoyl, 2-hydroxycinnamoyl, 3-hydroxycinnamoyl, 4-hydroxycinnamoyl, hydrocinnamoyl, 4-formylcinnamoyl, 3-hydroxy-4-methoxycinnamoyl, 4-hydroxy-3-methoxycinnamoyl, 2-carboxycinnamoyl, 3,4-dihydroxyhydrocinnamoyl, 3,4-dihydroxycinnamoyl, *trans*-Cinnamoyl, ( $\pm$ )-mandelyl, ( $\pm$ )-mandelyl-( $\pm$ )-mandelyl, glycolyl, 3-formylbenzoyl, 4-formylbenzoyl, 2-formylphenoxyacetyl, 8-formyl-1-naphthoyl, 4-(hydroxymethyl)benzoyl, 3-hydroxybenzoyl, 4-hydroxybenzoyl, 5-hydantoinacetyl, L-hydroorotyl, 2,4-dihydroxybenzoyl, 3-benzoylpropanoyl, ( $\pm$ )-2,4-dihydroxy-3,3-dimethylbutanoyl, DL-3-(4-hydroxyphenyl)lactyl, 3-(2-hydroxyphenyl)propionyl, 4-(2-hydroxyphenyl)propionyl, D-3-phenyllactyl, 3-(4-hydroxyphenyl)propionyl, L-3-phenyllactyl, 3-pyridylacetyl, 4-pyridylacetyl, isonicotinoyl, 4-quinolinecarboxyl, 1-isoquinolinecarboxyl and 3-isoquinolinecarboxyl.

35

- 77 -

and B is a carboxy-terminal modifying group selected from the group consisting of an amide group, an alkyl amide group, an aryl amide group and a hydroxy group.

2. A compound having the structure:

5



wherein (Xaa) is a peptidic structure selected from the group consisting of D-Leu-D-Val-D-Phe-D-Phe, D-Leu-D-Val-D-Phe-phenethylamide, D-Leu-D-Val-D-Tyr-D-Phe, D-Leu-D-Val-D-IodoTyr-D-Phe, D-Leu-D-Val-D-Phe-D-Tyr, D-Leu-D-Val-D-Phe-D-IodoTyr, D-Leu-D-Val-D-Phe-D-Ala, D-Ala-D-Val-D-Phe-D-Phe-D-Leu, D-Leu-D-Val-D-Tyr-D-Phe-D-Ala, D-Leu-D-Val-D-IodoTyr-D-Phe-D-Ala, D-Leu-D-Val-D-Phe-D-Tyr-D-Ala, D-Leu-D-Val-D-Phe-D-IodoTyr-D-Ala, D-Phe-D-Phe-D-Val-D-Leu, D-Ala-D-Phe-D-Phe-D-Val, D-Ala-D-Phe-D-Phe-D-Val-D-Leu, D-Ala-D-Phe-D-Phe-D-Leu-D-Leu, D-Leu-D-Phe-D-Phe-D-Val-D-Leu, D-Phe-D-Phe-D-Phe-D-Val-D-Leu, D-Phe-D-Phe-D-Phe-D-Leu-D-Val, D-Phe-D-Phe-D-Phe-D-Phe-D-Leu and D-Ala-D-Phe-D-Phe-D-Phe-D-Leu.

20

A is an amino-terminal modifying group selected from the group consisting of cholyl, lithocholyl, hyodeoxycholyl, chenodeoxycholyl and ursodeoxycholyl; and

B is a carboxy-terminal modifying group selected from the group consisting of an amide group, an alkyl amide group, an aryl amide group and a hydroxy group.

3. The compound of claim 3, wherein A is selected from the group consisting of lithocholyl, hyodeoxycholyl, chenodeoxycholyl and ursodeoxycholyl.

4. A compound having the structure: 3,5-Dihydroxy-2-Naphthoyl- (D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine)-NH<sub>2</sub>.

5. A compound having the structure: 3,7-Dihydroxy-2-Naphthoyl- (D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine)-NH<sub>2</sub>.

6. A compound having the structure: 4-Hydroxybenzoyl- (D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine)-NH<sub>2</sub>.

35



- 78 -

7. A compound having the structure: 4-Hydroxyphenylacetyl- (D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine)-NH<sub>2</sub>.

8. A compound having a structure selected from the group consisting of:

- 5 Diphenylacetyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-NH<sub>2</sub>;  
Triphenylacetyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-NH<sub>2</sub>;  
Isobutanoyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-phenylalanine-NH<sub>2</sub>;  
4-Methylvaleryl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-phenylalanine-NH<sub>2</sub>;  
10 3-Hydroxyphenylacetyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-NH<sub>2</sub>;  
3,5-Dihydroxy-2-naphthoyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-NH<sub>2</sub>;  
3,5-Dihydroxy-2-naphthoyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine-NH<sub>2</sub>;  
15 2-Hydroxyphenylacetyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-NH<sub>2</sub>;  
3,4-Dihydroxycinnamoyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-NH<sub>2</sub>;  
(±)-Mandelyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-NH<sub>2</sub>;  
(±)-Mandelyl-(±)-Mandelyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine-NH<sub>2</sub>;  
20 Glycolyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine-NH<sub>2</sub>;  
Glycolyl-D-phenylalanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
D-alanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
D-leucine-D-valine-D-iodotyrosine-D-phenylalanine-D-alanine-NH<sub>2</sub>;  
D-alanine-D-phenylalanine-D-phenylalanine-D-leucine-D-leucine-NH<sub>2</sub>;  
25 D-leucine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
D-phenylalanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
D-phenylalanine-D-phenylalanine-D-phenylalanine-D-leucine-D-valine-NH<sub>2</sub>;  
D-phenylalanine-D-phenylalanine-D-phenylalanine-D-phenylalanine-D-leucine-NH<sub>2</sub>;  
D-alanine-D-phenylalanine-D-phenylalanine-D-phenylalanine-D-leucine-NH<sub>2</sub>;  
30 D-alanine-D-homophenylalanine-D-homophenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
D-leucine-D-phenylalanine-D-phenylalanine-D-phenylalanine-D-leucine-NH<sub>2</sub>;  
D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-leucine-NH<sub>2</sub>;  
D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-phenylalanine-NH<sub>2</sub>;  
35 D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-valine-NH<sub>2</sub>;

- 79 -

- Hyodeoxycholyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine-NH<sub>2</sub>;  
Chenodeoxycholyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine-NH<sub>2</sub>;  
Ursodeoxycholyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine-NH<sub>2</sub>;  
5 2,4-Dihydroxybenzoyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine-NH<sub>2</sub>;  
2-Hydroxycinnamoyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine-NH<sub>2</sub>;  
10 3-Hydroxycinnamoyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine-NH<sub>2</sub>;  
4-Hydroxycinnamoyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine-NH<sub>2</sub>;  
5-Hydantoinacetyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine-NH<sub>2</sub>;  
15 3,4-Dihydroxycinnamoyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine-NH<sub>2</sub>;  
3-Formylbenzoyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine-NH<sub>2</sub>;  
20 2-Formylphenoxyacetyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine-NH<sub>2</sub>;  
4-Hydroxy-3-methoxycinnamoyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine-NH<sub>2</sub>;  
3,5-Dihydroxy-2-naphthoyl-D-alanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
25 3,4-Dihydroxycinnamoyl-D-alanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
(±)-Mandelyl-D-alanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
4-Hydroxycinnamoyl-D-alanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
30 Glycolyl-D-alanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
3,4-Dihydroxycinnamoyl-D-leucine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
Glycolyl-D-phenylalanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;

- 80 -

- DL-3-(-4-Hydroxyphenyl)Lactyl-D-leucine-D-valine-D-phenylalanine-D-phenylalanine-D-alanine-NH<sub>2</sub>;  
D-3-Phenyllactyl-D-alanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;
- 5 L-3-Phenyllactyl-D-alanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
3-(2-Hydroxyphenyl)propionyl-D-alanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
4-(2-Hydroxyphenyl)propionyl-D-alanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;
- 10 D-3-Phenyllactyl-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
3-(2-Hydroxyphenyl)propionyl-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
3-(4-Hydroxyphenyl)propionyl-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;
- 15 Hydrocinnamoyl-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
4-Hydroxybenzoyl-D-alanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
D-3-Phenyllactyl-D-phenylalanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;
- 20 Hydrocinnamoyl-D-phenylalanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
4-Hydroxybenzoyl-D-leucine-D-phenylalanine-D-phenylalanine-D-phenylalanine-D-leucine-NH<sub>2</sub>;  
Acetyl-D-leucine-D-phenylalanine-D-phenylalanine-D-phenylalanine-D-leucine-NH<sub>2</sub>;
- 25 3,7-Dihydroxy-2-naphthoyl-D-leucine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
4-Hydroxyphenylacetyl-D-leucine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;
- 30 3,7-Dihydroxy-2-naphthoyl-D-phenylalanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
4-Hydroxyphenylacetyl-D-phenylalanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
3,7-Dihydroxy-2-naphthoyl-D-Pyridylalanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;

- 81 -

- 4-Hydroxybenzoyl-D-Pyridylalanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
3-Pyridylacetyl-D-alanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
Isonicotinoyl-D-alanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
5 H-Glycyl-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
H-Glycyl-D-alanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
4-Pyridylacetyl-D-alanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
4-Quinolinecarboxyl-D-alanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
10 1-Isoquinolinecarboxyl-D-alanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
3-Isoquinolinecarboxyl-D-alanine-D-phenylalanine-D-phenylalanine-D-valine-D-leucine-NH<sub>2</sub>;  
4-Hydroxybenzoyl-D-leucine-D-valine-D-phenylalanine-D-tyrosine-D-alanine-NH<sub>2</sub>;  
15 4-Hydroxybenzoyl-D-leucine-D-valine-D-tyrosine-D-phenylalanine-D-alanine-NH<sub>2</sub>;  
and  
4-Hydroxybenzoyl-D-leucine-D-valine-D-tyrosine-D-tyrosine-D-alanine-NH<sub>2</sub>.

9. A pharmaceutical composition comprising a therapeutically effective  
20 amount of the compound of claim 1-8 and a pharmaceutically acceptable carrier.

10. A method for inhibiting aggregation of natural  $\beta$ -amyloid peptides,  
comprising contacting the natural  $\beta$ -amyloid peptides with the compound of claim 1-8  
such that aggregation of the natural  $\beta$ -amyloid peptides is inhibited.

25

11. A method for detecting the presence or absence of natural  $\beta$ -amyloid  
peptides in a biological sample, comprising:

contacting a biological sample with the compound of claim 1-8, wherein the  
compound is labeled with a detectable substance; and

30 detecting the compound bound to natural  $\beta$ -amyloid peptides to thereby detect  
the presence or absence of natural  $\beta$ -amyloid peptides in the biological sample.

12. The method of claim 11, wherein the  $\beta$ -amyloid modulator compound  
and the biological sample are contacted *in vitro*.

35

- 82 -

13. The method of claim 11, wherein the  $\beta$ -amyloid modulator compound is contacted with the biological sample by administering the  $\beta$ -amyloid modulator compound to a subject.

5           14. The method of claim 11, wherein the compound is labeled with radioactive technetium or radioactive iodine.

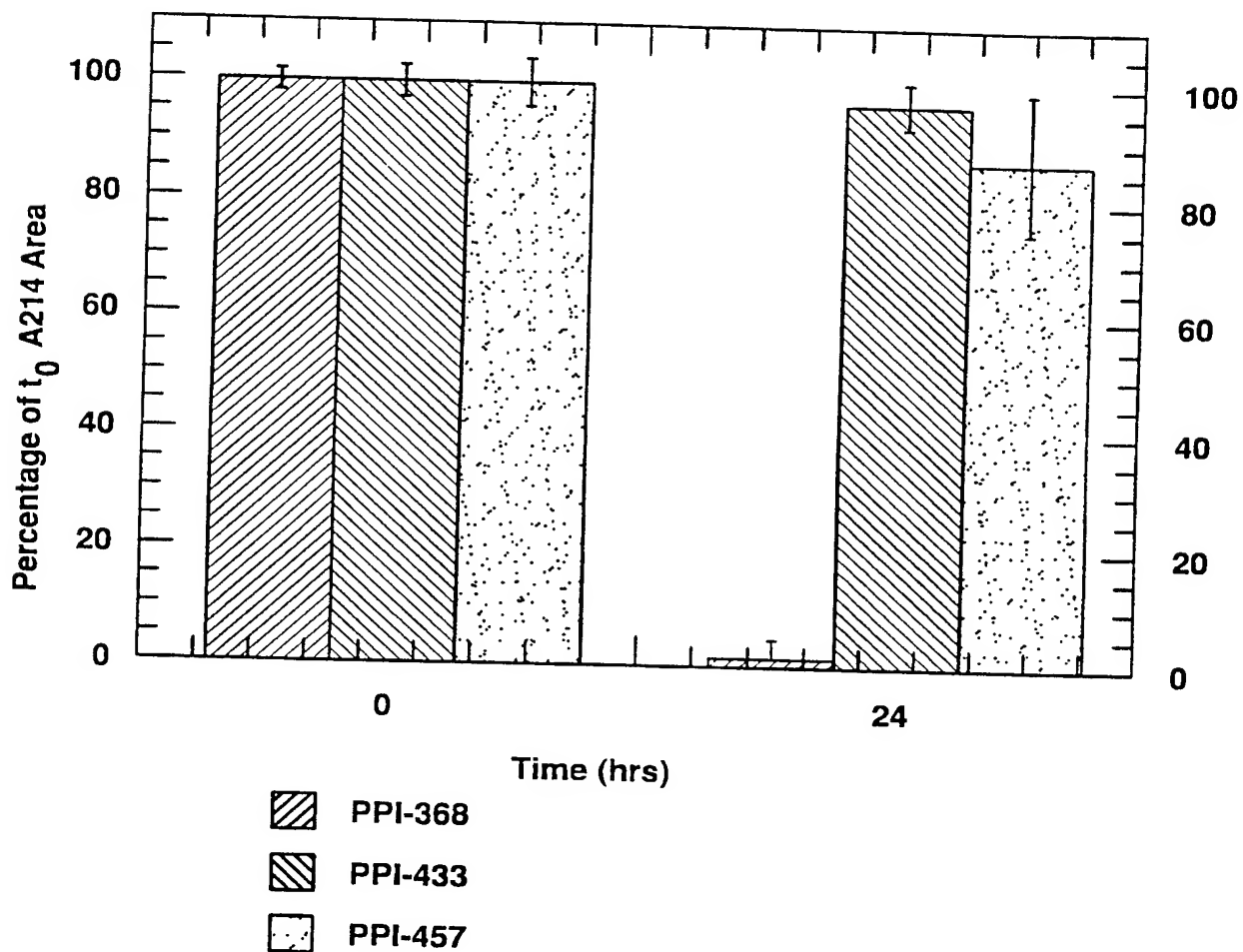
15. Use of the compound of claim 1-8 in therapy.

10           16. Use of the compound of claim 1-8 in the manufacture of a medicament for the treatment of a disorder associated with  $\beta$ -amyloidosis.

17. The use of claim 16, wherein the disorder is Alzheimer's disease.

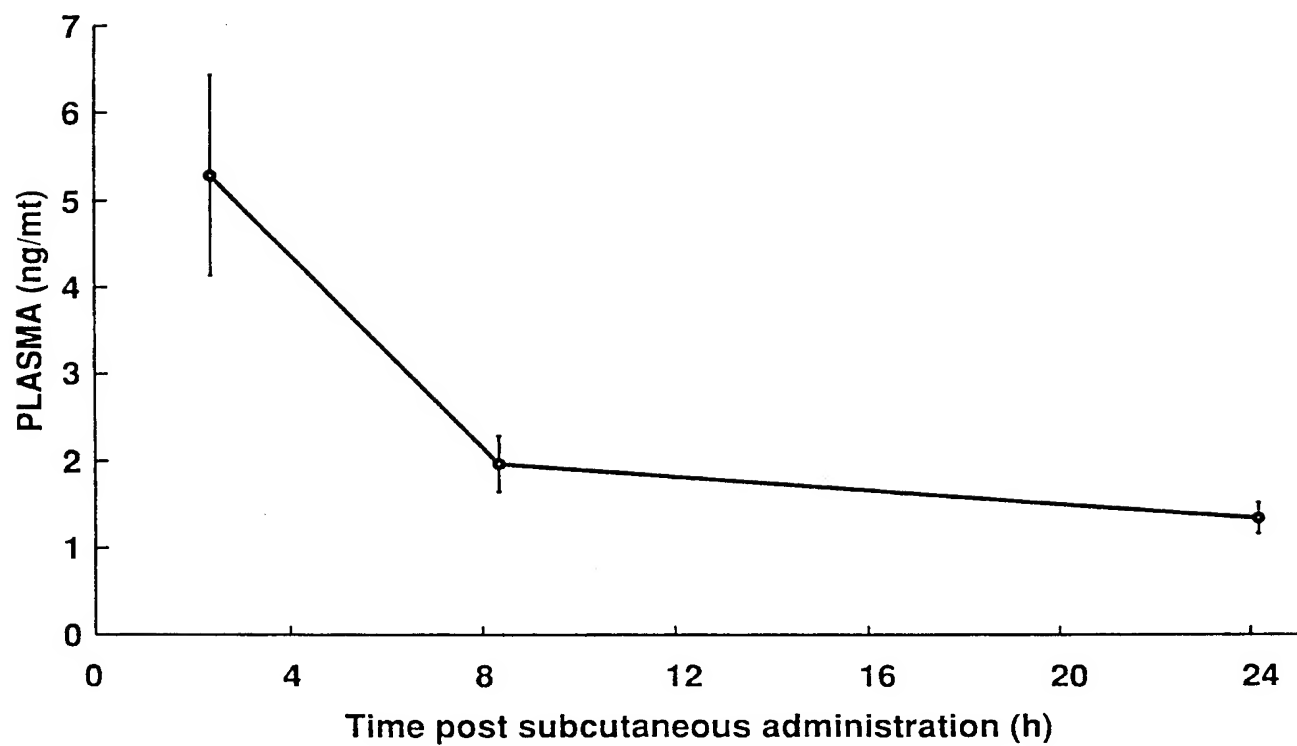
1 / 4

FIG. 1



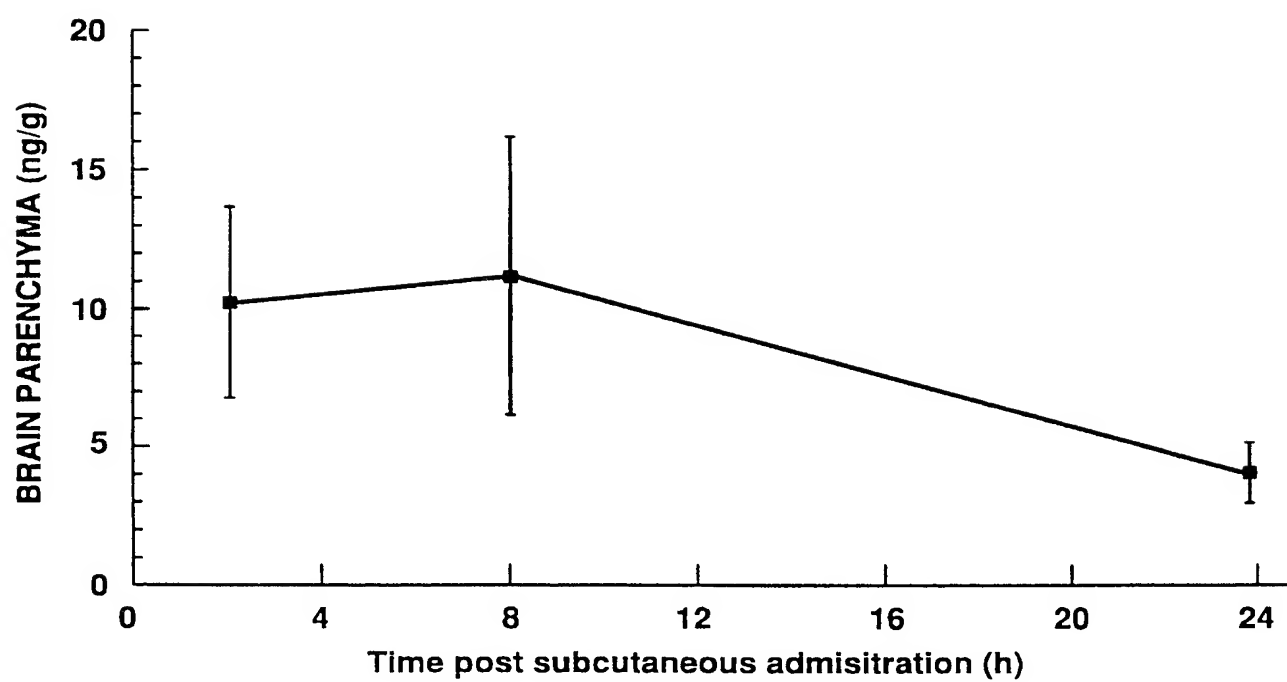
2 / 4

FIG. 2



3 / 4

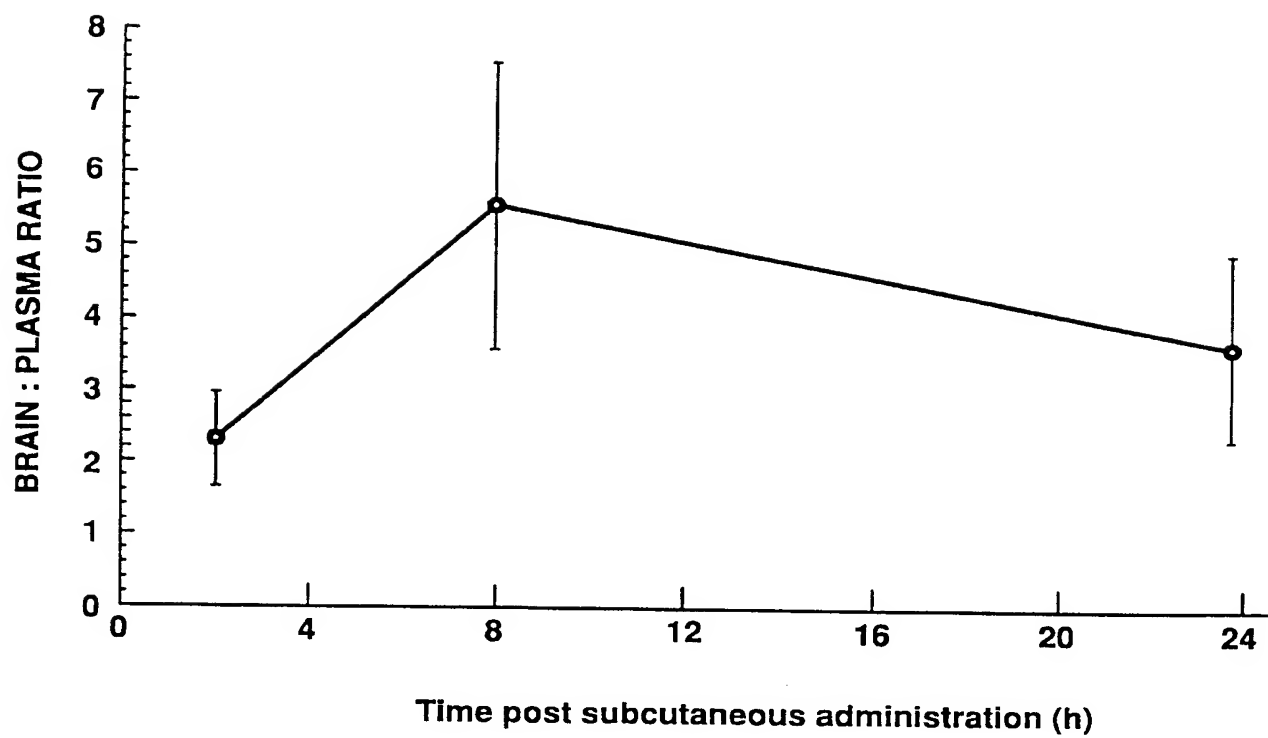
FIG. 3





4 / 4

FIG. 4



# INTERNATIONAL SEARCH REPORT

Interr. .nal Application No  
PCT/US 97/15166

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K14/47 A61K38/17 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TJERNBERG E.A.: "Arrest of $\beta$ -amyloid fibril formation by a pentapeptide ligand" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 15, 12 April 1996, MD US, pages 8545-8548, XP002050226 see the whole document ---	1-17
A	FLOOD E.A.: "Topography of a binding site for small amnesic peptides deduced from structure-activity studies: relation to amnesic effect of amyloid B protein" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, January 1994, WASHINGTON US, page 380-384 XP002050227 see the whole document --- -/--	1-17

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### ° Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

15 December 1997

Date of mailing of the international search report

21.01.98

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Groenendijk, M

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/15166

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WOOD E.A.: "Prolines and amyloidogenicity in fragments of the Alzheimer's peptide B/A4" BIOCHEMISTRY, vol. 34, no. 3, 24 January 1995, EASTON, PA US, pages 724-730, XP002050228 see the whole document ---	1-17
A	WO 93 11772 A (UNIV PRINCETON) 24 June 1993 see the whole document ---	1-17
P,X	SOTO E.A.: "Inhibition of Alzheimer's amyloidosis by peptides that prevent B-sheet conformation" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 226, no. 3, 24 September 1996, ORLANDO, FL US, pages 672-680, XP002050229 The whole document; see especially pp.677-678; fig.5 ---	1-17
P,X	TJERNBERG E.A.: "Controlling amyloid B-peptide fibril formation with protease-stable ligands" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 19, 9 May 1997, MD US, pages 12801-12806, XP002050230 see the whole document ---	1-17
P,Y	WO 97 21728 A (KAROLINSKA INNOVATIONS AB; NORDSTEDT CHRISTER (SE); NAESLUND JAN ()) 19 June 1997 See especially ex.5 ---	1-17
P,Y	WO 96 28471 A (PHARMACEUTICAL PEPTIDES INC) 19 September 1996 see the whole document -----	1-17

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 97/15166

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  

Although claims 10,11,13-15 are directed to a method of treatment of or to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 97/15166

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9311772 A	24-06-93	US 5338837 A	16-08-94
		AU 3278593 A	19-07-93
		BR 9206927 A	21-11-95
		CA 2117332 A	24-06-93
		EP 0618800 A	12-10-94
		HU 70743 A	30-10-95
		JP 7503708 T	20-04-95
		NO 942165 A	01-08-94
		PL 171131 B	28-03-97
		US 5455335 A	03-10-95
		US 5693769 A	02-12-97
		US 5627270 A	06-05-97
		US 5571795 A	05-11-96
-----			
WO 9721728 A	19-06-97	AU 1072897 A	03-07-97
-----			
WO 9628471 A	19-09-96	AU 5252496 A	02-10-96
-----			



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 38/00, C07K 2/00, 4/00, 5/00, 7/00, 14/00, 16/00, 17/00, C12Q 1/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/22120</b>  <b>(43) International Publication Date:</b> 28 May 1998 (28.05.98)
<b>(21) International Application Number:</b> PCT/US97/21116 <b>(22) International Filing Date:</b> 19 November 1997 (19.11.97)  <b>(30) Priority Data:</b> 60/031,169 19 November 1996 (19.11.96) US  <b>(71) Applicants (for all designated States except US):</b> THE WISTAR INSTITUTE OF ANATOMY & BIOLOGY [US/US]; 3601 Spruce Street, Philadelphia, PA 19104 (US). TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Suite 300, 3700 Market Street, Philadelphia, PA 19104 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> OTVOS, Laszlo [US/US]; 801 Mockingbird Lane, Audubon, PA 19403 (US). HOFFMANN, Ralf [DE/US]; 6812 Drexelbrook Drive, Drexel Hill, PA 19026 (US). LEE, Virginia, M-Y. [US/US]; 2005 Pine Street, Philadelphia, PA 19103 (US).  <b>(74) Agents:</b> KODROFF, Cathy, A. et al.; Howson and Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US).		<b>(81) Designated States:</b> AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> DIAGNOSTIC AND THERAPEUTIC REAGENTS FOR ALZHEIMER'S DISEASE  <b>(57) Abstract</b>  <p>A synthetic multiphosphorylated peptide derived from the <math>\tau</math> protein of the paired helical filaments is described. The Alzheimer disease specificity is provided by the presence of phosphate moieties on nearby serine and threonine residues, and the reverse-turn structure of the multiphosphorylated protein fragment. The multiphosphorylated peptide is useful as an antigen and a binding partner for identifying inhibitor compounds which interact with the peptide and the hyperphosphorylated tau protein, including Alzheimer's specific antibodies. The resulting antibodies are useful diagnostically and therapeutically. The inhibitors that specifically bind to multiphosphorylated tau peptides and proteins are useful for eliminating abnormally hyperphosphorylated tau.</p>		

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## DIAGNOSTIC AND THERAPEUTIC REAGENTS FOR ALZHEIMER'S DISEASE

This invention was supported by the National Institutes of Health grants AG10670 and AG10210. The US government has certain rights in this invention.

### Field of the Invention

The present invention relates generally to compositions useful in diagnosis of Alzheimer's disease, methods of producing same, and structural requirements for inhibitor design.

### Background of the Invention

The major histopathological abnormalities that characterize the brains of patients with Alzheimer's disease (AD) as well as those of older individuals with Down syndrome include excesses of neurofibrillary tangles (NFT), and senile plaques (SP) (K. S. Kosik, *Science* 256:780-783 (1992); V.M.-Y. Lee and J. Q. Trojanowski, *Curr. Opin. Neurobiol.*, 2:653-656 (1992); D. J. Selkoe, *Trends Neurosci.*, 16:403-409 (1993)]. The NFT are composed of paired helical filaments (PHF) that are biochemically and structurally distinct from the amyloid fibrils in SP. PHF and the straight filaments that dominate ultrastructural images of neurofibrillary lesions are likely formed from hyperphosphorylated forms of the low molecular weight microtubule-associated  $\tau$  protein, known as PHF- $\tau$  [M. Goedert, *Trends Neurosci.*, 16:460-465 (1993); J. Q. Trojanowski et al, *Clin. Neurosci.*, 1:184-191 (1994)]. In contrast, the major subunit proteins of amyloid fibrils are  $\beta$ -amyloid peptides (A $\beta$ ). They are about 42 amino acids long, and are derived from one or more larger, alternatively spliced amyloid precursor proteins (APP), encoded by a gene on chromosome 21 [J. Hardy, *J. NIH Res.*, 5:46-49 (1993); M. Mullan and F. Crawford, *Trends Neurosci.*, 16:409-414 (1993)]. SP are sites at which abundant accumulations of both PHF and amyloid fibrils converge (Trojanowski et al., 1994, cited above). Despite the presence of APP mutations in rare cases of familial AD, the abundance of



NFT in the brain correlates better with dementia severity in AD than the density of amyloid plaques [D. W. Dickson et al, Neurobiol. Aging, 13:179-189 (1991); P. A. Arriagada et al, Neurology, 42:631-639 (1992)].

5 A major prerequisite for evaluating therapeutic and/or prevention strategies of AD, the availability of valid biological markers of AD is still lacking. In this regard, it is significant that  $\tau$  levels are increased in the cerebrospinal fluid (CSF) of AD patients and that the CSF  $\tau$  levels correlate with clinical measures of dementia severity [C. Mock et al, Ann. Neurol., 37:414-415 (1995)]. The assay used the very sensitive monoclonal antibody (mAb) AT120 for detection of  $\tau$ . Unfortunately, mAb  
10 AT120 detects normal  $\tau$  and PHF- $\tau$  just as well. Most recently, tau was detected in humans. The development and use of more specific antibodies that are both highly sensitive, and can distinguish between normal and deranged  $\tau$ , may further enhance the discriminative properties of such assays and seems likely to be the focus of research in the forthcoming years.

15 The sites of abnormal phosphorylation of PHF- $\tau$  and the structural changes abnormal phosphorylation bring, are two of the most studied and debated areas of the current AD research. Functionally,  $\tau$  binds to tubulin and PHF- $\tau$  does not [C. W. Scott et al, J. Neurosci. Res., 33:19-29 (1992); Biernat et al., Neuron, 11:153-163 (1993)]. The lack of the microtubule binding of PHF- $\tau$  is usually attributed to  
20 hyperphosphorylation as tubulin binding can be restored by dephosphorylating PHF- $\tau$  [P. Seubert et al., J. Biol. Chem., 270:18917-18922 (1995)]. There is some concern that, after several years of intense analysis of the phosphorylation status of  $\tau$ , there is still no compelling evidence that phosphorylation of  $\tau$  has any causal relation to the formation of PHFs. The views opposing hyperphosphorylated  $\tau$  as a primary reason  
25 for PHF production come from the observation that constructs corresponding roughly to the microtubule-binding repeat region of  $\tau$  can form synthetic PHFs. A similar self-assembly occurs with chemical cross-linking of dimers. In both cases, there is no need for phosphorylation of the protein. The absence of abnormal hyperphosphorylation of  $\tau$  in intracellular tangles of AD was also noted by studying the staining of NFT with  
30 supposedly phosphate-specific mAbs, such as AT8, AT180, AT270, SMI31, Alz50

and BR133 [W. Bondareff et al., *J. Neuropathol. Exp. Neurol.*, 54:657-663 (1995)]. In reality, however, these antibodies may not be ultimately specific for PHF (Goedert et al., 1994, cited above). The conclusive test whether an antibody is truly specific for PHF is the antibody's ability to label fetal  $\tau$  and biopsy-derived  $\tau$ . Fetal  $\tau$  and biopsy-  
5 derived  $\tau$  were shown to contain more phosphate groups than normal  $\tau$  [T. J. Singh et al., *Arch. Biochem. Biophys.*, 328:43-50 (1996); Garver et al., *J. Neurosci. Res.*, 44:12-20 (1996)]. Dephosphorylation of PHF- $\tau$  can be very fast at some sites (Seubert et al., 1995, cited above), giving rise to the possibility of unrealistic immunoreactions with antibodies directed against these sites. Biopsy-derived  $\tau$  refers  
10 to  $\tau$  preparations from healthy individuals when the protein is isolated from the brain tissue seconds after acquisition, and  $\tau$  is not exposed to the extremely fast phosphatase actions that occur in the tissues even post-mortem.

An increasing amount of evidence suggests that mAbs obtained after immunization with PHF- $\tau$  recognize multiphosphorylated protein fragments. The  
15 major recognition site of mAb PHF-1 is phosphorylated Ser396, but the recognition is increased when Ser404 is also phosphorylated [Otvos et al., *J. Neurosci. Res.*, 39:669-673 (1994)]. Another often used antibody to PHF- $\tau$  is AT8 (Biernat et al., cited above). This mAb binds specifically to phosphorylated Ser202 and Thr205. As the previous investigations used singly phosphorylated synthetic peptides or mutated  
20 protein constructs to identify the binding sites of these mAbs, the close-to-minimal or minimal epitopes and exact phosphate requirements of the antibodies have not been characterized.

What is needed in the art are diagnostic antibodies that distinguish PHF- $\tau$  from other  $\tau$  forms such as biopsy-derived  $\tau$ , fetal  $\tau$  and normal  $\tau$ . Also  
25 needed is an understanding of how phosphorylation changes the conformation of tau and its fragments in order to design compounds that are able to binding hyperphosphorylated tau and remove it once it is formed.

Summary of the Invention

In one aspect, the invention provides a synthetic multiphosphorylated peptide derived from the tau protein of the paired helical filaments (PHF) associated with Alzheimer's disease. The multiphosphorylated peptide is useful as an immunogen  
5 for preparing an antibody specific for Alzheimer's disease.

In another aspect, the present invention provides a method for generating one or more antibodies useful in the specific diagnosis of Alzheimer's disease. The method involves at least the step of administering to a mammal a synthetic multiphosphorylated peptide of the invention.

10 In yet another aspect, the present invention provides one or more antibodies generated according to the method of the invention. The resulting antibodies are characterized by specificity for the tau protein of the paired helical filaments associated with Alzheimer's disease.

In a further aspect, the present invention provides a method of treating  
15 a patient with Alzheimer's disease comprising the step of administering to the patient a reagent of the invention. Preferably, this reagent is an antibody.

In still a further aspect, the present invention provides a method of treating a patient with Alzheimer's disease comprising the step of administering to the patient a multiphosphorylated peptide of the invention. Suitably, this generates anti-  
20 peptide antibodies that are able to eliminate abnormally hyperphosphorylated tau peptide.

In yet a further aspect, the present invention provides for the use of a multiphosphorylated peptide of the invention in designing and identifying compounds useful in the diagnosis and treatment of Alzheimer's disease.

25 Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

### Brief Description of the Figures

Fig. 1 illustrates the selection of PHF-specific antibodies by ELISA. The diagonally hatched bars indicate the recognition of the newly developed mAbs against normal  $\tau$ . The cross-hatched bars indicate the recognition of the mAbs against A68, putative AD-specific proteins, and purified forms of PHF (Lee et al., 1991). The single asterisks indicate the mAbs selected for further studies. PHF11 was selected because high background was obtained and (based on the first screening) its PHF-specificity could not be established (double asterisk).

Fig. 2A illustrates the binding of mAb PHF-1 to different tau preparations on Western blot. Lanes 1 and 2 are normal adult autopsy-derived  $\tau$ , lanes 3 and 4 are fetal  $\tau$ , lanes 5 and 6 are PHF- $\tau$ .

Fig. 2B illustrates the binding of mAb PHF-6 to different tau preparations on Western blot. Lanes 1 and 2 are normal adult autopsy-derived  $\tau$ , lanes 3 and 4 are fetal  $\tau$ , lanes 5 and 6 are PHF- $\tau$ .

Fig. 2C illustrates the binding of mAb PHF-13 to different tau preparations on Western blot. Lanes 1 and 2 are normal adult autopsy-derived  $\tau$ , lanes 3 and 4 are fetal  $\tau$ , lanes 5 and 6 are PHF- $\tau$ .

Fig. 2D illustrates the binding of mAb PHF-27 to different tau preparations on Western blot. Lanes 1 and 2 are normal adult autopsy-derived  $\tau$ , lanes 3 and 4 are fetal  $\tau$ , lanes 5 and 6 are PHF- $\tau$ .

Fig. 3 illustrates peptide recognition of mAb PHF-6. The ELISA plate was coated with 8 ng - 1  $\mu$ g antigens dissolved in water. Solid line: unphosphorylated peptide  $\tau$  224-240 [SEQ ID NO: 8]; dashes: peptide phosphorylated on Thr231 [SEQ ID NO: 9]; dots: peptide phosphorylated on Ser235 [SEQ ID NO: 10]; dots and dashes: peptide phosphorylated on both residues [SEQ ID NO: 11]. Undiluted supernatant of cells producing the antibody were used.

Fig. 4 illustrates peptide recognition of mAb PHF-27. The ELISA plate was coated with 4 ng- 2  $\mu$ g antigens dissolved in water. Solid line: unphosphorylated peptide  $\tau$  224-240 [SEQ ID NO: 8]; dashes: peptide phosphorylated on Thr231 [SEQ ID NO: 9]; dots: peptide phosphorylated on Ser235 [SEQ ID NO: 10]; dots and

dashes: peptide phosphorylated on both residues [SEQ ID NO: 11]. Undiluted supernatant was used.

Fig. 5 provides the identification of the epitope for mAb AT10. The ELISA plate was coated with 70 ng - 5  $\mu$ g monophosphorylated or diphosphorylated 208-222 peptides dissolved in water. The mAb was diluted to 1:300. Only the Thr 212, Ser214 diphosphorylated peptide was recognized by mAb.

Fig. 6A illustrates the dependence of antibody binding upon the conformation of the antigens, where the primary antibody is PHF-6. The peptides were applied to the ELISA plate in water or TFE, and dried to the plastic. Solid line: Thr231 monophosphorylated peptide applied from water [SEQ ID NO: 9]; dots: the same peptide applied from TFE. Dashes: Thr231, Ser235 diphosphorylated peptide applied from water [SEQ ID NO: 11]; dots and dashes: the same peptide applied from TFE. Many of the curves show the characteristic "pro-zone" antibody binding at high antigen loads, illustrating the close-to-optimal antigen-antibody interactions.

Fig. 6B illustrates the dependence of antibody binding upon the conformation of the antigens, where the primary antibody is PHF-27. The ELISA was performed as described in the legend to Fig. 6A.

Fig. 7 illustrates the dependence of antibody binding upon the conformation of the antigens where the primary antibodies are TG3 and AT180. The curves are as follows: Solid lines represent the Thr231, Ser235 double phosphorylated peptide [SEQ ID NO: 11] applied from TFE; dots and dashes represent the same peptide applied from water. Dashes represent the Thr231 phosphorylated peptide [SEQ ID NO: 9] applied from TFE; dots represent the same peptide applied from water. The ELISA was performed as described in the legend to Fig. 6A.

Fig. 8A provides the CD spectra of  $\tau$  224-240 peptides in water. Solid line: unphosphorylated peptide [SEQ ID NO: 8]; dots: Thr231 phosphorylated peptide [SEQ ID NO: 9]; dashes: Ser235 phosphorylated peptide [SEQ ID NO: 10]; dots and dashes: diphosphorylated peptide [SEQ ID NO: 11].

Fig. 8B provides the CD spectra of  $\tau$  224-240 peptides in TFE. Solid line: unphosphorylated peptide [SEQ ID NO: 8]; dots: Thr231 phosphorylated

peptide [SEQ ID NO: 9]; dashes: Ser235 phosphorylated peptide [SEQ ID NO: 10]; dots and dashes: diphosphorylated peptide [SEQ ID NO: 11].

Fig. 9A illustrates low energy conformers of the peptide backbone of unphosphorylated peptide  $\tau$  224-240 [SEQ ID NO: 8]. High dielectric constant was used to mimic solvent effects (78 for water for TFE). Unconstrained simulated annealing calculations were performed as described herein. The structures were overlaid automatically based on the similarity of all backbone atoms.

Fig. 9B illustrates low energy conformers of the peptide backbone of unphosphorylated peptide  $\tau$  224-240 [SEQ ID NO: 8]. Low dielectric constant was used to mimic solvent effects (4.5 for TFE). Unconstrained simulated annealing calculations were performed as described herein. The structures were overlaid automatically based on the similarity of all backbone atoms.

Fig. 9C illustrates low energy conformers of the peptide backbone of monophosphorylated peptide Thr231 [SEQ ID NO: 9]. High dielectric constant was used to mimic solvent effects (78 for water). Unconstrained simulated annealing calculations were performed as described herein. The structures were overlaid automatically based on the similarity of all backbone atoms.

Fig. 9D illustrates low energy conformers of the peptide backbone of monophosphorylated peptide Thr231 [SEQ ID NO: 9]. Low dielectric constant was used to mimic solvent effects (4.5 for TFE). Unconstrained simulated annealing calculations were performed as described herein. The structures were overlaid automatically based on the similarity of all backbone atoms.

Fig. 9E illustrates low energy conformers of the peptide backbone of diphosphorylated analogue of peptide  $\tau$  224-240 [SEQ ID NO: 11]. High dielectric constant was used to mimic solvent effects (78 for water for TFE). Unconstrained simulated annealing calculations were performed as described herein. The structures were overlaid automatically based on the similarity of all backbone atoms.

Fig. 9F illustrates low energy conformers of the peptide backbone of a diphosphorylated analogue of peptide  $\tau$  224-240 [SEQ ID NO: 11]. Low dielectric constant was used to mimic solvent effects (4.5 for TFE). Unconstrained simulated

annealing calculations were performed as described herein. The structures were overlaid automatically based on the similarity of all backbone atoms.

Fig. 10 demonstrates the specificity of anti-phosphopeptide antisera to unphosphorylated [SEQ ID NO: 8] and Thr231, Ser235 double phosphorylated [SEQ ID NO: 11]  $\tau$  224-240 peptides as well as normal  $\tau$  and PHF- $\tau$  proteins. The double phosphorylated [SEQ ID NO: 11]  $\tau$  224-240 peptide was co-linearly synthesized with a turn-inducing spacer (Ala-Gly-Ala) and sequence 31D [SEQ ID NO: 25], and C3H mice were immunized with the chimeric peptide. After booster immunization, the anti-phosphopeptide mouse sera were analyzed for recognition of normal  $\tau$  and its fragment as well PHF- $\tau$  and its fragment.

Fig. 11A illustrates binding of  $\alpha$ - $\omega$  diamines and polyamines to the Thr231 monophosphorylated analog [SEQ ID NO: 9], to the Ser235 monophosphorylated analog [SEQ ID NO: 10] and to the Thr231, Ser235 double phosphorylated analog [SEQ ID NO: 11] of the  $\tau$  224-240 peptide. A modified ELISA protocol was used, in which the reduction of phosphorylation site-specific anti-PHF- $\tau$  antibody recognition was measured after the phosphopeptides were mixed with the various diamines and polyamines. The assay for the Thr231 monophosphorylated peptide [SEQ ID NO: 9] was developed with monoclonal antibody PHF-6 (specific for phosphorylated Thr231), for the Ser235 monophosphorylated peptide [SEQ ID NO: 10] with monoclonal antibody TG4 (specific for phosphorylated Ser235) and for the Thr231, Ser235 double phosphorylated peptide [SEQ ID NO: 11] with monoclonal antibody MC5 (specific for double phosphorylated Thr231 and Ser235).

Fig. 11B illustrates binding of  $\alpha$ - $\omega$  diamines and polyamines to PHF- $\tau$  protein and to the Thr231, Ser235 double phosphorylated  $\tau$  224-240 peptide [SEQ ID NO: 11]. A modified ELISA assay was used, as described in Fig. 11A. The assay was developed with monoclonal antibody PHF-6 (specific for phosphorylated Thr231), with monoclonal antibody TG4 (specific for phosphorylated Ser235) and with monoclonal antibody MC5 (specific for double phosphorylated Thr231 and Ser235).

### Detailed Description of the Invention

The present invention provides multiphosphorylated peptides useful in generating antibodies specific for the tau protein of the paired helical filaments found in the brain of patients with Alzheimer's disease. The inventors have found that  
5 antibodies specific for Alzheimer's disease-originated tau protein seem to recognize a reverse-turn conformation between the phosphate sites. The multiphosphorylated peptides are able to bind multivalent compounds. It is this observation which permitted the inventors to find that the multiphosphorylated peptides and antibodies generated are useful diagnostically and/or therapeutically, as well as for drug design  
10 for Alzheimer's disease.

#### I. Tau ( $\tau$ ) Peptide Fragments

The multiphosphorylated peptides of the invention are desirably derived from a fragment of the  $\tau$  protein of the paired helical filaments which are present in the brains of Alzheimer's patients.

15 The  $\tau$  protein may be obtained from a variety of sources, include adult-autopsy tissue, fetal sources, or biopsy-derived tissue, or may be synthesized using techniques known to those of skill in the art. All references to  $\tau$  amino acid residues follow the numbering scheme provided in Goedert et al, Neuron, 3:519-526 (1989), which is incorporated by reference herein.

20 Desirably, the  $\tau$  protein fragment which may serve as a template for constructing the peptides of the invention is between about 4 and about 20 amino acids in length, more preferably about 15 to about 20 amino acids in length. Exemplary  $\tau$  protein fragments include the fragment spanning about amino acid residue 207 to about 222 of  $\tau$ , about amino acid residue 224 to about 240 of  $\tau$ , and  
25 the fragment spanning about amino acid residues 390 to about 408 of  $\tau$ . The sequences of these fragments are illustrated in Table 1 below. The present invention is not limited to these fragments. One of skill in the art can readily select other appropriate fragments given the guidance provided herein and the knowledge in the art.



According to the invention, a desired  $\tau$  protein fragment is modified to contain, or synthesized to contain, at least two phosphorylated amino acids. In a preferred embodiment, the amino acids are serines or threonines, or a combination thereof. In a particularly preferred embodiment, the serine(s) and/or threonine(s) are separated by one to up to six amino acids, i.e., they may be immediately adjacent or separated by as many as six amino acids. For instance, exemplary phosphorylated peptides of the invention, which are derived from amino acids 207-222 of  $\tau$  [SEQ ID NO: 1], include: GSRSR(T)P(S)LPTPPTRE [SEQ ID NO: 5], GSRSR(T)PSLP(T)PPTRE [SEQ ID NO: 6], and GSRSRTP(S)LP(T)PPTRE [SEQ ID NO: 7], where the parentheses ( ) indicate a phosphorylated amino acid. Another exemplary phosphorylated peptide of the invention includes KKVAVVR(T)PPK(S)PSSAK [SEQ ID NO: 11], which is derived from amino acids 224-240 of  $\tau$  [SEQ ID NO: 8]. Still other exemplary phosphorylated peptides of the invention include AEIVYK(S)PVV(S)GDTSPRHL [SEQ ID NO: 17], AEIVYK(S)PVVSGD(T)SPRHL [SEQ ID NO: 18], AEIVYK(S)PVVSGDT(S)PRHL [SEQ ID NO: 19], AEIVYKSPVV(S)GD(T)SPRHL [SEQ ID NO: 20], AEIVYKSPVV(S)GDT(S)PRHL [SEQ ID NO: 21], and AEIVYKSPVVSGD(T)(S)PRHL [SEQ ID NO: 22], all of which are derived from amino acids 390-408 of  $\tau$  [SEQ ID NO: 12]. Other suitable phosphorylated peptides of the invention, including those with more than two phosphorylated amino acids may be readily prepared by one of skill in the art based on these and other  $\tau$  peptide fragments.

### III. Production/Synthetic Peptides

Where desired, the  $\tau$  protein fragments and the phosphorylated peptides of the invention are produced using synthetic techniques. For example, the peptides may be generated using a commercially available automatic synthesizer according to standard procedures. In this manner, phosphoserine or phosphothreonine residues may be incorporated in the course of synthesis.

Alternatively, other standard techniques may be utilized. See, e.g., Merrifield, J. Amer. Chem. Soc., 85:2149-2154 (1963).

Although less desired, in view of the size of the  $\tau$  peptide fragments and the phosphorylated peptides of the invention, these fragments and peptides may  
5 be prepared by known recombinant DNA techniques by cloning and expressing, within a host microorganism or other cell, a DNA fragment carrying a coding sequence for a multiphosphorylated peptide, antibody or antibody fragment of the invention. Suitably, the expression system is selected from among those which are capable of adding the required number of phosphate groups, e.g., insect cells infected with  
10 baculovirus. Coding sequences for the peptides (or antibodies) of the invention can be prepared synthetically or can be derived from viral RNA by known techniques, or from available cDNA-containing plasmids. Thus, the invention also encompasses nucleic acid sequences encoding the peptides, antibodies and antibody fragments, of the invention. These nucleic acid sequences are useful not only for recombinant  
15 production methods, but may themselves be used in the diagnostic and therapeutic methods described herein.

Systems for cloning and expressing the phosphorylated peptides of the invention in various cells, including, for example, bacterial, mammalian, yeast and insect cells, and suitable vectors may be readily selected from among known and  
20 available from private and public laboratories and depositories and from commercial vendors. Currently, the most preferred host is a mammalian cell such as Chinese Hamster ovary cells (CHO) or COS-1 cells. These hosts may be used in connection with poxvirus vectors, such as vaccinia or swinepox. The selection of other suitable host cells and methods for transformation, culture, amplification, screening and  
25 product production and purification can be performed by one of skill in the art by reference to known techniques. See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981). The antibodies of the invention and fragments thereof are also amenable to recombinant production techniques, such as those described herein. Another preferred system includes the baculovirus expression system and vectors. Bacterial  
30 expression may also be desired.

When produced by conventional recombinant means, the phosphorylated peptides of the invention may be isolated either from the cellular contents by conventional lysis techniques or from cell medium by conventional methods, such as chromatography. See, e.g., Sambrook et al., Molecular Cloning. A Laboratory Manual., 2d Edit., Cold Spring Harbor Laboratory, New York (1989).

#### IV. Production of Antibodies/Inhibitors

The phosphorylated peptides of the invention are useful in generating both polyclonal and monoclonal antibodies or for testing the binding of other bivalent compounds. Advantageously, these antibodies are specific for the phosphorylated peptides from which they are generated, i.e., the antibodies do not bind to normal  $\tau$  fragments or other normal proteins.

Specific antisera (polyclonal antibodies) may be generated using known techniques. See, Sambrook, cited above, Chapter 18, generally, incorporated by reference. Similarly, monoclonal antibodies of the invention may be produced by conventional methods, including the Kohler and Milstein hybridoma technique, recombinant techniques, such as described by Huse et al, Science, 246:1275-1281 (1988), or any other techniques known to the art.

The invention further encompasses functional fragments of the antibodies of the invention, including, Fab,  $F_v$ , and  $F(ab')_2$  fragments, synthetic molecules containing the binding site of the antibodies of the invention, and the complementarity determining regions (CDRs) thereof. Further, these functional fragments may be used in the production of recombinant antibodies, including bifunctional antibodies, chimeric antibodies, and humanized antibodies, which preferably retain the antigen binding specificity of the antibodies of the invention. Such recombinant antibodies may be constructed and produced according to known techniques [see, e.g., S. D. Gillies et al, J. Immunol. Meth., 125:191-202 (1989); and G. E. Mark and E. A. Pladlan, "Humanization of Monoclonal Antibodies", The Handbook of Experimental Pharmacology, Vol. 113, Chapter 4, pp. 105-133, Springer-Verlag (June, 1994)]. These functional fragments and recombinant

antibodies may be used for a variety of purposes, including any of those described herein for the antibodies of the invention.

In general, polyclonal antisera, monoclonal antibodies and other antibodies which bind to a phosphorylated peptide as the antigen (Ab1) are useful to  
5 identify epitopes of  $\tau$ , to separate  $\tau$  from contaminants in living tissue (e.g., in chromatographic columns and the like), and in general as research tools and for the production of other types of antibodies described above. Anti-idiotypic antibodies (Ab2) are useful for binding  $\tau$  and thus may be used in the treatment of Alzheimer's disease. Other uses as research tools and as components for separation of  $\tau$  from  
10 other contaminants of living tissue, for example, are also contemplated for these antibodies.

#### V. Diagnostic applications

Advantageously, the present invention provides reagents and methods useful in detecting and diagnosing Alzheimer's disease. Thus, the phosphorylated  
15 peptides and antibodies of the invention may be useful as diagnostic reagents. These reagents may optionally be labelled using diagnostic labels, such as radioactive labels, colorimetric enzyme label systems and the like conventionally used in diagnostic or therapeutic methods. Alternatively, the – or C-terminus of a phosphorylated peptide of the invention may be tagged with a detectable label which can be recognized by a  
20 specific antisera or another binding mechanism, e.g., biotin-streptavidin. The terminal labels include those detected by fluorimetric, colorimetric, etc., methods. The reagents may be used in diagnosis of Alzheimer's disease. For example, ELISA or other immunological methods can be used for the detection of hyperphosphorylated tau protein in cerebrospinal fluid (CSF) or blood; these methods make use of the  
25 antibodies of the invention. The selection of the appropriate assay format and label system is within the skill of the art and may readily be selected by a skilled artisan.

Thus, the present invention provides methods for the detection of Alzheimer's disease. The methods involve contacting a selected mammalian tissue,

particularly brain tissue, or CSF, or serum, in a selected assay format based on the binding or hybridization of the reagent to the sample.

#### VI. Therapeutic Applications

Compositions and methods useful for the treatment of conditions associated with Alzheimer's disease are provided. The therapeutic compositions of the invention may be formulated to contain a phosphorylated peptide or antibody of the invention, or a fragment thereof. In one desirable embodiment, an antibody of the invention is used for passive immunotherapy. In another embodiment, the multiphosphorylated peptide is used as an immunogen for active immunotherapy. In still another embodiment, a compound (e.g., a bivalent reagent described below) identified through use of the multiphosphorylated peptide of the invention) is used for treatment of Alzheimer's disease. The therapeutic composition desirably contains 0.01  $\mu$ g to 10 mg peptide, protein, or reagent. These compositions may contain a pharmaceutically acceptable carrier. Suitable carriers are well known to those of skill in the art and include, for example, saline. Alternatively, such compositions may include conventional delivery systems into which protein of the invention is incorporated. Optionally, these compositions may contain other active ingredients, e.g., chemotherapeutics. The dose, timing and mode of administration of these compositions may be determined by one of skill in the art. Such factors as the age and condition of the patient, may indicate increasing or decreasing the dose, frequency or mode of administration of the therapeutic compositions of the invention. Generally, administration in a site-directed manner and is repeated as needed. Such therapy may be administered in conjunction with conventional therapies.

#### VII. Drug Design

The phosphorylated peptides of the present invention may also be used in the design, screening and development of simple chemical compounds, proteins, or complex biopolymers which have utility as diagnostic and/or therapeutic drugs for Alzheimer's disease.

As one example, a compound capable of bivalent binding to the multi-phosphorylated peptide or hyperphosphorylated tau protein of the invention and either preventing or enhancing its biological activity may be a useful drug component for the treatment or prevention of Alzheimer's disease. In particular, the ability of some  
5 diamines and polyamines to selectively bind to the abnormally hyperphosphorylated tau peptides and proteins is demonstrated herein. Other suitable compounds may similarly bind concomitantly to two phosphate groups on the tau peptides or proteins described herein, i.e., phosphorylated tau peptides and proteins where the phosphate groups are separated by a distance of about 5 to 12Å. Such a compound may be  
10 obtained using known techniques from a peptide library, an organic chemical library (including a library of diamines or polyamines), or derived from a variety of sources, including, for example, extracts of supernatants from culture of bioorganisms, extracts from organisms collected from natural sources, known chemical compounds, and mixtures thereof.

15 Utilizing the phosphorylated peptides or antibodies of the invention, conventional assays and techniques may be utilized for the screening and development of drugs capable of competitively binding to these peptides and proteins. An example of one suitable method involves the incubation of a test compound and the phosphorylated peptide or an antibody of the invention which may be immobilized on  
20 a solid support. Alternatively, the incubation may be performed fully in solution (e.g., fluorescence polarization). Still other conventional methods of drug screening can involve employing a suitable computer program to determine compounds having similar or a complementary chemical structure to that of a phosphorylated peptide or antibody of the invention and screening those compounds for competitive binding.

25 Thus, through use of such methods, the present invention is anticipated to provide compounds capable of interacting with the phosphorylated peptides of the invention, and either enhancing or decreasing its biological activity, as desired. Such compounds are encompassed by this invention.

It should be understood that one of skill in the art may readily select the type of conventional screening method most desirable, as well as the reagent of this invention, e.g., the phosphorylated peptide, antibody or fragment thereof.

Desirably, these designed drugs, or drugs identified through these  
5 screening assays, are specific for Alzheimer's disease and do not interact with normal proteins, including normal  $\tau$ . Such specificity can be assessed using known techniques, including those illustrated in the examples below. For example, specificity of binding can be determined by comparing the binding of the identified compound (e.g., an inhibitor) to a multiphosphorylated peptide of the invention and to a single  
10 phosphorylated version of the same amino acid stretch or negative control unphosphorylated or multi-phosphorylated sequences. Particularly suitable multiphosphorylated peptides useful for the drug design are GRSR(S)P(S)LPTPPTRE [SEQ ID NO: 5] and KKVAVVR(T)PPK(S)PSSAK [SEQ ID NO: 11]. Particularly suitable control sequences include  
15 ADEV(S)A(S)LAKQGL (corresponding to amino acids 429-442 from the C-terminal end of  $\tau$ , SEQ ID NO: 23) and multiphosphorylated sequences from normal proteins, such as those from neurofilaments (e.g., EEKGK(S)PVPK(S)PVEEKG, SEQ ID NO: 24).

These examples illustrate the preferred methods for preparing the  
20 phosphorylated peptides and the antibodies of the invention. These examples are illustrative only and do not limit the scope of the invention.

#### Example 1 - Immunization with PHF

Highly purified PHF- $\tau$  preparations were prepared as described [V.M.-Y. Lee et al, *Science*, 251:675-678 (1991)]. Immunization with PHF- $\tau$  and fusion with  
25 mouse myeloma cells SP2/0-Ag14 followed previously published protocols (Lee et al, (1987)).

Immunization of mice with PHF $\tau$  yielded a number of mAbs that were tested for their antigen recognition by Western-blot and ELISA, as described in Example 2 below. The antigens included adult autopsy-derived  $\tau$ , fetal  $\tau$ , and PHF $\tau$ .

As controls, four existing mAbs were added to the mAb pool: AT10 [H.E. Mercken et al, EMBO J., 11:1593-1597 (1992)]; PHF-1 [S. G. Greenberg, et al, J. Biol. Chem., 267:564-569 (1992)], AT180 [M. Goedert et al, Biochem. J., 301:871-877 (1994)], and TG3 [Vincent et al, J. Cell. Biol., 132:413-425 (1996)]. PHF-1 and AT180 have  
5 been reported to label fetal  $\tau$  and PHF  $\tau$ , but not autopsy-derived normal adult  $\tau$  (Matsuo et al, 1994). In contrast, mAb AT10 labels only PHF- $\tau$  on Western blot (Matsuo et al, 1994) and TG3 labels predominantly PHF-tau [Vincent et al, 1996, cited above].

#### Example 2 - Western Blotting and Enzyme-linked immunosorbant assay (ELISA)

10 The epitopes of 12 monoclonal antibodies (mAb) raised against PHF (as described in Example 1) that recognized PHF- $\tau$ , but not normal  $\tau$  on Western-blot and enzyme-linked immunosorbent assay (ELISA) were mapped. The epitope analysis, together with known binding sites of previously published and widely used mAbs, revealed Ser214, Thr231 and Ser396 as immunodominant phosphorylated  
15 amino acids in PHF- $\tau$ . All mAbs recognizing these phosphorylated sites, with the exception of PHF-27, also labeled fetal  $\tau$  and biopsy-originated  $\tau$ . In contrast to previous findings, these data indicate that Ser214 can be phosphorylated in fetal  $\tau$ .

##### A. ELISA

Direct ELISA was applied to look for regular or conformation-  
20 sensitive epitopes [E. Lang, et al, J. Immunol. Meth., 170:103115 (1994); L. Otvos, Jr. and G. I. Szendrei, Enzyme-linked immunosorbent assay of peptides. In: Neuropeptide Protocols (Levine, B., and Williams, C., eds) Human Press, Totowa, pp. 269-275 (1996)]. Briefly, binding of 0.04-5  $\mu$ g amounts of peptide or phosphopeptide antigens was tested with 1/50 to 1/3600 dilutions of mAbs on 96-well  
25 Nunc Immuno MaxiSorp plates. The antigens were dissolved in water or trifluoroethanol (TFE), and dried down to the plates at 37°C overnight. All subsequent steps were performed according to general ELISA protocols [J. W. Goding, Monoclonal Antibodies: Principles and Practice. Academic Press, Orlando (1986)]. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin was



used as a secondary antibody at 1/1000 dilution. Color development was made with o-phenylenediamine, stopped with 1M aqueous  $H_3PO_4$ , and absorption was measured at 450 nm. Purified autopsy-derived normal  $\tau$ , and PHF- $\tau$  at 5  $\mu$ g/ml concentration were used as controls, and processed for ELISA as discussed above.

5                    B.     Western-blot

Western-blotting using autopsy-derived normal adult  $\tau$ , fetal  $\tau$ , PHF  $\tau$  was performed as previously described [Matsuo et al, (1994), cited above]. Briefly, nitrocellulose replicas of gels containing electrophoretically separated  $\tau$  samples were prepared from 10% sodium dodecyl sulfate-polyacrylamide gels and  
10                    probed with the primary antibodies. MAb binding was detected as previously described (Lee et al, 1991, cited above). Protein concentrations in the samples were determined using bicinchonic acid as a dye reagent with bovine serum albumin as the standard (Smith et al, 1985). The amount of protein loaded depends on the different  $\tau$  preparations which varied from 1.5 to 12 $\mu$ g. The protein concentration was adjusted  
15                    to obtain approximately equally strong immunoreactivity (if binding occurs at all) of various samples, based on binding of the same preparations to phosphorylation-independent antibody standards T14/46 (Matsuo et al, 1994, cited above). Each experiment was repeated at least three times.

Example 3 - Peptide Synthesis

20                    In this study, highly purified synthetic peptides, as well as single and double phosphorylated analogues, were used to identify the binding sites of four of the frequently used antibodies, AT180 (Goedert et al., 1994, cited above), PHF-1 (Greenberg et., 1992, cited above), and AT10 [Mercken et al, 1992, cited above] and TG3 [Vincent et al, 1996, cited above], and of 12 mAbs to full-sized PHF proteins,  
25                    described herein. Since phosphate transfer is a common side-reaction during phosphopeptide synthesis and storage, the integrity of the antigens was continuously verified.

Peptides were synthesized on Milligen 9050 and Rainin PS3 automatic synthesizers using 9-fluorenylmethoxycarbonyl amino acids according to standard

procedures [G.B. Fields and R. L. Noble, Int. J. Pept. Protein Res., **35**: 161-214 (1990)]. Phosphoserine or phosphothreonine residues were incorporated as Fmoc-Ser/Thr(PO<sub>3</sub>HBzl)-OH [T. Wakamiya et al, Chem. Lett., 1099-1102 (1994)] purchased from Novabiochem, Ltd. Peptides and phosphopeptides were detached  
5 from the solid support with trifluoroacetic acid (TFA) and they were purified by reversed-phase high performance liquid chromatography (RP-HPLC) using an aqueous acetonitrile gradient elution system containing 0.1% TFA as an ion pairing reagent. The integrity of the peptides and phosphopeptides was verified by mass spectroscopy. Table 1 lists the synthetic peptides. An asterisk indicates a  
10 phosphorylated amino acid. These peptides are designated by SEQ ID NOS: 1-22, respectively. In other words,  $\tau$  207-222 is SEQ ID NO: 1 and the doubly phosphorylated  $\tau$  390-408 (403P, 404P) is SEQ ID NO: 22.

Table 1

Synthetic peptides and their characterization.

	Peptides	Sequence [min]	Retention time	Calculated mass [M+H] <sup>+</sup>	Observed mass [M+H] <sup>+</sup>
5	τ207-222	GSRSRTPSLPTPTRE	23.8	1739	1739
	212P	T*	23.6	1819	1821
	214P	S*	23.6	1819	1819
10	217P	T*	24.2	1819	1820
	212P,214P	T*S*	23.1	1899	1898
	212P,217P	T* T*	23.9	1899	1900
	214P,217P	S* T*	23.6	1899	1898
	τ224-240	KKVAVVRTPPKSPSSAK	19.7	1780	1780
15	231P	T*	20.0	1860	1860
	235P	S*	19.8	1860	1860
	231P,235P	T* S*	19.9	1940	1940
	τ390-408	AEIVYKSPVVSGDTSRHL	25.5	2055	2055
	396P	S*	24.1	2135	2134
20	400P	S*	24.9	2135	2134
	403P	T*	25.1	2135	2136
	404P	S*	25.3	2135	2136
	396P,400P	S* S*	24.5	2215	2214
	396P,403P	S* T*	26.6	2215	2217
25	396P,404P	S* S*	24.3	2215	2214
	400P,403P	S* T*	25.3	2215	2215
	400P,404P	S* S*	27.3	2215	2214
	403P,404P	T*S*	32.0	2215	2214

30 The new mAbs detect PHF-τ but not normal τ. Epitope analysis revealed immunodominant phosphoserine and phosphothreonine residues in PHF. One of the newly developed mAbs, PHF-27, labels only PHF-τ, insignificantly labels fetal τ, and does not label biopsy-originated τ. What additional factors the PHF-specific mAbs (e.g. PHF-27) would detect was studied by studying the conformation of the epitopes.

Example 4 - Antibody Binding

The antibodies prepared as described in Example 3 above were tested for their binding to three synthetic peptide families, which were prepared as described below. All three peptide groups contained unphosphorylated, monophosphorylated  
5 and diphosphorylated peptides.

Among the newly developed mAbs, only mAb PHF-27 binds specifically only to PHF- $\tau$  and not fetal, autopsy or biopsy-derived  $\tau$ . These results indicate that like mAb AT10, mAb PHF-27 is a PHF-specific antibody.

As show herein, the peptide recognition of mAb PHF-27 was markedly  
10 increased when both the primary site Thr231 and the subsite Ser235 were phosphorylated. This conformation-sensitive ELISA, supported by circular dichroism measurements and molecular modeling of the phosphorylated peptide epitope (see examples 5 and 6 below), indicated that while the other antibodies recognized just the phosphorylated immunodominant amino acids, mAb PHF-27 recognized both the  
15 presence of the phosphate group and the conformation of the multiphosphorylated antigen. These data suggest that it is possible to develop true PHF-specific mAbs, but due to the limited epitope repertoire of the full protein, it is more beneficial to use designed, multiphosphorylated peptide immunogens, such as those of the invention.

Of the new 12 mAbs tested, five recognized phosphoamino acids in  
20 one of the three peptide families above. In fact, three major immunodominant residues could be identified, i.e., phosphorylated Ser214, Thr231, and Ser396 (Table 2).

22

Table 2

Immunodominant phosphoamino acids in PHF- $\tau$ .

	Monoclonal antibody	Recognized primary phosphate site	Secondary phosphate site
5	New antibodies		
	PHF-20	Ser214	Thr217
	PHF-6	Thr231	
	PHF-27	Thr231	Ser235
10	PHF-13	Ser396	
	PHF-47	Ser396	
	Existing antibodies used in this study		
	AT10	Thr212 and Ser214 together	
	AT180	Thr231	
15	Literature data on the same sites <sup>1</sup> :		
	M4	Thr231 <sup>2</sup>	
	PHF-1	Ser396	Ser404 <sup>3</sup>
	AD-2	Ser396	Ser404
20	<sup>1</sup> This list contains only mAbs generated by immunizing with PHF		
	<sup>2</sup> M. Hasegawa et al., <u>FEBS Lett.</u> , <u>384</u> :25-30 (1996).		
	<sup>3</sup> Otvos et al, 1994.		
	<sup>4</sup> Buee-Scherrer et al, <u>Mol. Brain Res.</u> , <u>39</u> :79-88 (1996).		

Mass spectrometry indicate Thr231 and Ser235, two phosphorylated residues of PHF- $\tau$  in the AT180 epitope region: [Morishima-Kawashima et al., *J. Biol. Chem.*, 270:823-829 (1995)]. The  $\tau$  224-240 peptide [SEQ ID NO: 8], the two

25 monophosphorylated analogues (231P and 235P) [SEQ ID NOS: 9 and 10,

respectively] and the diphosphorylated peptide (231P,235P) [SEQ ID NO: 11] were synthesized in a way which places the phosphoamino acids in the middle of the molecule (Table 1). These peptides were made long enough to cover the entire epitope and possess some secondary structure, but short enough to allow preparation with the highest possible purity and not to have more than two conformational elements. MAb AT180 did not bind to unphosphorylated peptide  $\tau$  224-240 [SEQ ID NO: 8] or to the phosphorylated version 235P [SEQ ID NO: 10], but did bind to the same peptide phosphorylated at Thr231 [SEQ ID NO: 9] and to the diphosphorylated peptide 231P, 235P [SEQ ID NO: 11] (Table 2). Once Thr231 was phosphorylated, the antigen recognition of mAb AT180 was not increased after phosphorylation of Ser235. MAb AT180 bound to the Thr231 phosphorylated peptide very strongly. As little as 40ng phosphopeptide could be clearly detected at antibody dilution of 1:3600.

The antigen recognition pattern of new antibody mAb PHF-6 was completely identical to that of mAb AT180. MAb PHF-6 did not recognize the unphosphorylated [SEQ ID NO: 8], or Ser235 phosphorylated [SEQ ID NO: 10]  $\tau$  224-240 peptide, and recognized the Thr231 phosphorylated [SEQ ID NO: 9], and diphosphorylated peptides [SEQ ID NO: 11] equally well (Fig. 3). In contrast to these antibodies, the antigen recognition of mAb PHF-27 showed a different pattern. This mAb did not bind to the unphosphorylated  $\tau$  224-240 peptide [SEQ ID NO: 8], as expected. Like mAb PHF-6 and AT180, mAb PHF-27 recognized phosphorylated Thr231 [SEQ ID NO: 9] as a main site and did not recognize phosphorylated Ser235 [SEQ ID NO: 10]. When both Thr231 and Ser235 were phosphorylated [SEQ ID NO: 11], however, the antigen recognition was markedly increased, especially at low antigen amounts (Fig. 4).

MAbs PHF-47 and PHF-13, just like PHF-1, did not recognize unphosphorylated peptide  $\tau$  390-408 [SEQ ID NO: 12], or the same peptide phosphorylated at Ser400 [SEQ ID NO: 14], Thr403 [SEQ ID NO: 15] or Ser404 [SEQ ID NO: 16], but recognized the Ser396 phosphorylated peptide [SEQ ID NO: 13], and all diphosphorylated peptides containing phosphorylated Ser396 [SEQ ID NOS: 17-19]. None of the diphosphorylated peptides lacking phosphate group on

Ser396 [SEQ ID NOS: 20-22] were recognized by these mAbs (Table 2). MAb PHF-20.6 (subclone 6 of antibody 20) did not bind to the unphosphorylated peptide  $\tau$ 207-222 [SEQ ID NO: 1], or to the peptide phosphorylated at Thr212 (212P) [SEQ ID NO: 2]. Weak binding at high peptide loads was observed for  
5 monophosphorylated peptide 217P [SEQ ID NO: 4], and diphosphorylated peptide 212P, 217P [SEQ ID NO: 6]. The antibody binding was strong to Ser214 monophosphorylated peptide (214P) [SEQ ID NO: 3] (Table 2) but was not significantly increased after incorporation of a second phosphate group to Thr212 [SEQ ID NO: 5] or Thr 217 [SEQ ID NO: 7]. Thus, detailed epitope analysis  
10 indicated that mAb PHF-20.6 recognized phosphorylated Ser214 as a main site and very slightly recognized the surrounding subsites.

After identifying the immunodominant phosphoamino acids in PHF- $\tau$ , the three peptide families were used to identify the epitope of mAb AT10. As earlier attempts with monophosphorylated peptides and simple mutated proteins failed to  
15 identify the antigen binding site for this mAb, diphosphorylated peptides were concentrated upon. Indeed, no antibody binding was detected to any of the 12 unphosphorylated or monophosphorylated synthetic peptides, but the mAb bound to the antigens when both Thr212 and Ser214 were simultaneously phosphorylated (Fig. 5) [SEQ ID NO: 5]. Interestingly, this mAb failed to recognize the other two  
20 diphosphorylated versions of the same peptide, namely those that carried phosphate groups on Thr212 and Thr217 [SEQ ID NO: 6], or Ser214 and Thr217 [SEQ ID NO: 7]. These data, together with those obtained for mAbs PHF-27 clearly indicate that PHF specific epitope include doubly phosphorylated Thr212/Ser214 [SEQ ID NO: 5] and Thr231/Ser235 [SEQ ID NO: 11].

25 Which factors were recognized by mAb PHF-27 and TG3 recognized and mAbs PHF-6 and not by AT180 were then determined. All four antibodies saw phosphorylated Thr231 and did not see individually phosphorylated Ser235. To make this determination, the peptides that were recognized by the antibodies [i.e. 231P [SEQ ID NO: 9] and 231P,235P [SEQ ID NO: 11] (marked PP in the figure)] were  
30 plated for the ELISA from water and also from TFE. With mAbs PHF-6 and AT180

the antigen recognition was increased for both peptides when the conformation of the phosphopeptides was stabilized in TFE (Fig. 6A). This indicates that these antibodies recognized a peptide conformation that was not dominant in water but could be generated in TFE. When the antigen recognition of mAb PHF-27 was studied, the recognition was dramatically increased for the monophosphorylated peptide plated from TFE, but not for the already strong binder diphosphorylated peptide (Fig. 6B). This finding indicated that antigen recognition by the antibody was stronger toward the diphosphorylated peptide in aqueous solutions because mAb PHF-27 saw both the presence of the phosphate group at Thr231, and the conformation of the diphosphorylated peptide that it assumed in water. MAb PHF-6 and AT180 saw the phosphorylated Thr231 residue, but did not see the conformation of the diphosphorylated peptide in water. Mab PHF-27 stands out in Western-blot as well. The recognition of the 12 new mAbs was analyzed against normal  $\tau$ , fetal  $\tau$ , biopsy-derived  $\tau$  and PHF- $\tau$ . None of the antibodies recognized normal  $\tau$ , and all bound to PHF- $\tau$ . Nevertheless, all (including AT180 and PHF-1) but PHF-27 also bound to fetal  $\tau$  and biopsy-derived  $\tau$ . These results indicate that mAb PHF-27 is the a "true" PHF-specific antibody. Based on the conformation-sensitive ELISA assay, it is tempting to speculate that mAb PHF-27 recognized a specific multiphosphorylated peptide secondary structure. It is worth noting that similarly to mAb PHF27, the antigen recognition of AT10 did not increase when the diphosphorylated peptide antigen was plated from TFE, just the "pro-zone" binding was shifted to lower antigen amounts (data not shown). MAb PHF27 appeared to be weaker than the other anti-PHF antibodies [i.e. weaker peptide (compare Figs. 3 and 4) or protein (Fig. 1) recognition with the same antibody dilution compared, for example to mAb PHF-6 that recognizes the same primary site].

Monoclonal antibodies TG3 [Vincent et al., 1996 cited above] and AT180 [Goedert et al., 1994, cited above] recognize phosphorylated Thr231. To test the hypothesis that TG3 and AT180 may recognize different conformations of the protein, the binding of the four 224-240 synthetic peptides to these antibodies was analyzed by using our conformation-sensitive ELISA protocol (as described above).



Both the unphosphorylated [SEQ ID NO: 8] and the Ser235 phosphorylated [SEQ ID NO: 10] peptides were devoid of either TG3 or AT180 reactivity regardless of which solvent was used to apply the peptides to the plate (Fig. 7). AT180 was found to be nearly equally reactive with both the monophosphorylated Thr231 peptide [SEQ ID NO: 9] and the diphosphorylated peptide [SEQ ID NO: 11] regardless of the solvent conditions. Analysis of TG3 gave quite different results (Fig. 7). TG3 reactivity with the Thr231 phosphorylated peptide [SEQ ID NO: 9] was substantially increased after drydown from TFE, suggesting that stabilization of the local peptide conformation plays an important role in determining TG3 reactivity. Also, unlike AT180, TG3 was virtually unable to bind the diphosphorylated peptide [SEQ ID NO: 11] after drydown from water. The binding of mAb TG3 to the diphosphorylated peptide [SEQ ID NO: 11] was restored however, when the peptide was applied from TFE (Fig. 7), clearly showing that a certain conformation of the diphosphorylated peptide is needed for TG3 recognition. Based on the CD analysis performed in water and in TFE, this conformation is a reverse-turn.

In summary, by using a panel of monoclonal antibodies directed to PHF- $\tau$ , the presence of a phosphate group on Ser214 of fetal  $\tau$  [SEQ ID NO: 3], and the concomitant presence of phosphates on Thr231 and Ser235 of PHF- $\tau$  [SEQ ID NO: 11], were positively identified. MAb 20 labels fetal  $\tau$  but not normal  $\tau$  on Western-blot indicating that Ser214 carries a phosphate in immature brain. On the other hand, both Thr212 and Ser214 [SEQ ID NO: 5] needed to be phosphorylated for any recognition by mAb AT10 and both Thr231 and Ser235 [SEQ ID NO: 11] needed to be phosphorylated for full recognition by mAb PHF-27. MAb PHF-27 did not recognize normal  $\tau$  or biopsy-derived  $\tau$  and very insignificantly fetal  $\tau$ . MAb PHF-27 appears to be the a true PHF-specific antibody, and useful as a reagent for the development of AD-specific diagnostic markers. The binding of PHF-27 to PHF- $\tau$  in normal body fluids remains to be determined, albeit the low dilution of this antibody needed for ELISA and Western-blot indicates that PHF-27 may not be the final solution for this purpose. Based on the speculation of Goedert (cited above) Ser235 needs to be phosphorylated before Thr231 can be phosphorylated.  $\tau$  can be

phosphorylated at Thr231 by glycogen synthase kinase 3 only if Ser235 is already phosphorylated by mitogen activated protein (MAP) kinase. If this is indeed the reality, these results suggest that Ser loses its phosphate group during the brain development, but regains the phosphate during the development of AD. This explanation is consistent with both the current dynamic multiple kinase-phosphatase equilibrium theory that interprets normal microtubule assembly or abnormal PHF aggregation (Garver et al., 1996; Singh et al., 1996), and with the different sensitivity of these sites for phosphatase actions. While phosphorylated Ser235 is a preferential substrate site for protein phosphatase 2B [Gong et al, J. Neurochem., 62:803-806 (1994)], phosphorylated Thr231 is an unusually resistant residue to dephosphorylation [Hasegawa et al, J. Biol. Chem., 267:17047-17054 (1992)]. While Thr231 and Ser235 can be phosphorylated by a number of kinases *in vitro* [Morishima-Kawashima et al, (1995), cited above], Thr212 and Ser214 are substrates for specific kinases. Thr212 can only be phosphorylated by MAP kinase [Drewes et al, (1992), cited above] and brain kinase [Biernat et al, (1993), cited above], and Ser214 can only be phosphorylated by cyclic AMP-dependent protein kinase (cAMP-PK) [Scott et al, (1993), cited above]. Nevertheless, this site is a major target for cAMP-PK: 50% of phosphate incorporated into  $\tau$  by cAMP-PK is on Ser214 [Litersky et al, (1996), cited above]. MAP kinase transforms  $\tau$  to an AD-like immunological state and phosphorylation of Ser214 is one of the major factors responsible for decreasing the ability of  $\tau$  to nucleate microtubules [Brandt et al, (1994), cited above]. By requiring phosphate groups on both Thr212 and Ser214 for binding, mAb AT10 appeared to fortunately carry recognition sites for both AD features: lack of microtubule-binding and PHF-like immunoreactivity.

#### 25 Example 5 - Circular dichroism (CD)

Full size normal human  $\tau$  possesses only a negligible fraction of periodic ( $\alpha$ -helix or  $\beta$ -pleated sheet) conformations in solution [D. W. Cleveland et al, J. Mol. Biol., 116:227-247 (1977)] and this is independent of whether  $\tau$  is from the

brain or expressed bacterially. The secondary structure of the 22 synthetic peptides of Table 1 was studied.

CD spectra were taken on a Jasco J720 instrument at room temperature in a 0.2 mm path length cell. Double distilled water and spectroscopy grade trifluoroethanol were used as solvents. The peptide concentration was about 0.5 mg/ml, determined each time by quantitative RP-HPLC [G. I. Szendrei, et al, Eur. J. Biochem., 226:917-924 (1994)]. Curve smoothing was accomplished by the algorithm provided by JASCO. Mean residue ellipticity ( $[\Theta]_{MR}$ ) is expressed in degrees/dmole by using a mean residue weight of 110. Because the secondary structures of the peptides (especially phosphopeptides) provided by the current computer-assisted curve analyzing algorithms show a high error rate, the CD spectra evaluations were based on comparison with known peptide conformations [R. W. Woody, et al, Circular dichroism of peptides. In: The Peptides (Hruby, V.J., ed.) Academic Press, Orlando, pp. 15-114 (1985); L. Otvos, L., Use of circular dichroism to determine secondary structure of neuropeptides. In: Neuropeptide Protocols (Irvine, B., and Williams, C., eds) Human Press, Totowa, pp. 153-161 (1996)].

None of the 22 synthetic peptides shown in Table 1 exhibited any  $\beta$ -sheet component by CD. Instead, these peptides exhibited CD spectra indicative of various equilibria of unordered and turn conformers.

To explain the specificity of mAb PHF-27 and TG3 for PHF- $\tau$ , the conformation of the  $\tau$  224-240 peptides was studied in water and TFE. In water all four of the  $\tau$  224-240 peptides exhibited type U CD spectra, characteristic of peptides without conformational preferences (Woody, cited above). The lack of the small positive band around 220 nm indicated the presence of some ordered structures (Fig. 8A). Based on the CD of the peptides in TFE, this residual ordered structure was likely to be some turn conformation. The peptides exhibited type C CD spectra in TFE (Fig. 8B), characteristic of type I  $\beta$  turns [F. A. Smith and L. G. Pease, CRC Crit. Rev. Biochem., 8:315-399 (1980)] or mixtures of  $\beta$ -turns with dominant type I character [A. Perczel et al., Int. J. Pept. Protein Res., 41:223-236 (1993)]. Among the four peptides, the turn character was far stronger for the diphosphorylated

peptide, than for either of the unphosphorylated or the monophosphorylated variants. This in turn, may indicate that mAb PHF-27, as opposed to mAbs PHF 6 and AT1890, saw not only the presence of the phosphate group, but also the residual turn conformation of the diphosphorylated peptide in water. Antibody TG3 recognized the double phosphorylated peptide only when the antigen assumed turn structure. This may indicate that in PHF-tau, phosphorylated Thr231 and Ser235 define a reverse turn. This turn structure, specific for PHF-tau can be utilized in the design of inhibitors that would bind the abnormally multi-phosphorylated protein variant. To justify this assumption of the turn structure, simulated annealing calculations were run on the unphosphorylated, the Thr231 phosphorylated, and the diphosphorylated peptides. See Example 6.

#### Example 6 - Molecular modeling

Molecular modeling and conformational energy calculations were performed with the Quanta 4.1/CHARMm 22 software packages on a Silicon Graphics Indy R4600PC work station. All atom parameters were assigned automatically from the consistent valence force field library provided by the software. Dielectric constants (4.5 for TFE, and 78 for water) were employed in scaling electrostatic interactions [E. D. Getzoff et al, *Nature*, 306:287-290 (1983)]. To search the full conformational space which could be occupied by the molecules, a high-temperature molecular dynamics and simulated annealing protocol was implemented. The peptides were built up in type 1  $\beta$ -turn structure, and the energy was minimized by the steepest descents method. Molecular dynamics were run between 0-900 K. The molecules were heated in 6120 steps by 0.001 ps increments, equilibrated for 6000 steps and the simulation was run for 6000 steps. From the structures generated during the simulation, four low energy structures from various regions of the trajectory were randomly selected. The four structures were cooled to 300 K over 18 ps. Each structure was energy minimized as before the simulated annealing.

The modeling fully supported the spectroscopic and immunological findings. Low energy conformers in high dielectric constant environment were consistent with an increased population of turn structures of the diphosphorylated peptide (Fig. 9E) compared to the monophosphorylated (Fig. 9C) or the  
5 unphosphorylated analogue (Figs. 9A). Low dielectric constant environment stabilized the turn of the two phosphopeptides (Figs. 9D and 9F).

In addition to the selective recognition of the diphosphorylated peptide in water by MAb PHF-27 and the selective recognition of a turn structure of the diphosphorylated peptide by Mab TG3, two conclusions can be drawn.

10 1) The binding of the studied anti-PHF antibodies to the phosphopeptides is strongly dependent upon the length and/or the purity of the antigens as well as the dilution of the antibody preparation. Mabs PHF-20, PHF-47 and PHF-1 recognized phosphorylated amino acids other than the main sites (Ser214 for PHF-20 and Ser396 for PHF-1) in antigen preparations where the antigens were  
15 longer or less homogenous.

2) PHF- $\tau$  contains immunodominant phosphoserine and phosphothreonine residues in neighboring sites. The three most characteristic single sites are Ser214, Thr231 and Ser396 with participation of nearby phosphoaminoacids (Thr212, Thr217, Ser235 and Ser404). The mAbs against these phosphoamino acids  
20 were always dominant and the mAbs showed the same specificity. In turn, this suggests that the antibody repertoire that can be produced by using the full protein as immunogen is limited. Synthetic phosphopeptides, which are much shorter than the protein, and contain designed phosphoserines or phosphothreonines, display and present each of the phosphoamino acids much more efficiently than the long,  
25 heterogeneously phosphorylated protein does. Antibodies against designed phosphopeptides offer considerably increased variety and specificity to hidden linear or conformational epitopes that may be singly or multiply phosphorylated in PHF- $\tau$ , but not in normal  $\tau$ , fetal  $\tau$  or biopsy-originated  $\tau$ .

Example 7 - Generation of anti-phosphopeptide monoclonal antibodies specific for PHF- $\tau$ .

To obtain sensitive and selective antibodies to PHF- $\tau$ , a chimeric peptide construct was prepared, in which the immunodominant diphosphorylated  $\tau$  224-240 peptide [SEQ ID NO: 11] was co-linearly synthesized with a turn-inducing spacer (Ala-Gly-Ala) and the sequence 31D [SEQ ID NO: 25], which is a T-helper cell epitope of the rabies virus nucleoprotein [Ertl et al., *J. Virol.*, 63: 2885-2892, (1989)] in a T - B epitope orientation. Twenty  $\mu$ g of the chimeric construct was mixed with 50% complete Freund's adjuvant and was injected into the hind legs of C3H mice. Fourteen days later, the mice received a booster immunization of 20  $\mu$ g of the chimeric peptide in 50% incomplete Freund's adjuvant. Ten days after the booster immunization, the resulting antisera were analyzed for antigen specificity (Fig. 10). The sera exhibited the same diphosphorylated peptide and PHF- $\tau$  protein specificity as mAb PHF-27 on ELISA, but with considerably increased sensitivity.

Example 8 - Binding of the phosphopeptides and the PHF- $\tau$  protein to  $\alpha$ - $\omega$  diamines and polyamines.

The feasibility of the hypothesis was tested, i.e. that the abnormally hyperphosphorylated peptide and protein variants assume a structure that places the phosphate groups around a reverse-turn, and suitable molecules, which will selectively bind to the multiphosphorylated sequences, can be identified. Diamines can simultaneously interact with the two phosphate groups *via* ionic forces. To this end, the peptide and protein solutions were mixed with  $\alpha$ - $\omega$  diamines and polyamines and applied to the ELISA plate. To assess binding, a modified ELISA protocol was used, in which the reduction of phosphorylation site-specific anti-PHF- $\tau$  antibody recognition was measured after the phosphopeptides were mixed with the various diamines and polyamines. Three monoclonal antibodies were used, each specific to a given phosphate form of the antigens. The assay for the Thr231 monophosphorylated peptide [SEQ ID NO: 9] was developed with monoclonal antibody PHF-6 (specific for phosphorylated Thr231), for the Ser235 monophosphorylated peptide [SEQ ID

NO: 10] with monoclonal antibody TG4 (specific for phosphorylated Ser235) and for the Thr231, Ser235 double phosphorylated peptide [SEQ ID NO: 11] with monoclonal antibody MC5 (specific for double phosphorylated Thr231 and Ser325). Monoclonal antibodies TG4 and MC5 were provided by Dr. Peter Davies, Albert  
5 Einstein College of Medicine, Bronx, New York. The amino groups of the diamines were separated by 2-12 methylene groups, or by saturated or unsaturated ring systems. Polyamines included spermine and spermidine. Three diamines, 1,8-diamino-octane (#6), 1,10-diamino-decane (#7) and 1,12-diamino-dodecane (#8) bound to all three peptides (unspecific binding) (Fig. 11A). A number of diamines and  
10 polyamines bound only to the double phosphorylated peptide, most strongly the polyamines spermine (#16) and spermidine (#17) (Fig. 11A).

In the next step whether some of the diamines were able to bind the PHF- $\tau$  protein by using the same modified ELISA protocol was investigated. This assay is a complex assay, and therefore variable results were obtained. Nevertheless  
15 three amines (1,8-diamino-octane, spermine and spermidine) that bound to the peptides strongly, reduced the O.D. reading more than 0.1 (Fig. 11B), which value is generally accepted in ELISA as the sign of clearly positive binding. Significantly, the distance between the amino groups of the best binder diamines or between the amino groups at one side of the polyamines is around 10 Å, very close to the distance  
20 between the phosphate groups of our computer models of the double phosphorylated 224-240  $\tau$  peptide [SEQ ID NO: 11], when the phosphopeptide assumes a turn conformation. Based on these findings it is possible to identify simple compounds or biopolymers or their fragments that would selectively bind to the nearby phosphate groups of abnormally hyperphosphorylated PHF- $\tau$  protein and perhaps will be able to  
25 eliminate PHF- $\tau$  if its is once formed.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention are believed to be encompassed in the scope of the claims  
30 appended hereto.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Wistar Institute of Anatomy & Biology  
Trustees of the University of Pennsylvania  
Otvos, Laszlo  
Hoffmann, Ralf  
Lee, Virginia

(ii) TITLE OF INVENTION: Diagnostic and Therapeutic  
Reagents for Alzheimer's Disease

(iii) NUMBER OF SEQUENCES: 25

## (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Howson and Howson  
(B) STREET: Spring House Corporate Cntr., PO Box 457  
(C) CITY: Spring House  
(D) STATE: PA  
(E) COUNTRY: USA  
(F) ZIP: 19477

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: WO  
(B) FILING DATE:  
(C) CLASSIFICATION:

## (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 60/031,169  
(B) FILING DATE: 19-NOV-1996

## (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Kodroff, Cathy A.  
(B) REGISTRATION NUMBER: 33,980  
(C) REFERENCE/DOCKET NUMBER: WST77APCT



## (ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 215-540-9200

(B) TELEFAX: 215-540-5818

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly	Ser	Arg	Ser	Arg	Thr	Pro	Ser	Leu	Pro	Thr	Pro	Pro	Thr	Arg	Glu
1				5				10					15		

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 6

(D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Thr"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly	Ser	Arg	Ser	Arg	Thr	Pro	Ser	Leu	Pro	Thr	Pro	Pro	Thr	Arg	Glu
1				5				10					15		

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 8
- (D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Ser"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gly Ser Arg Ser Arg Thr Pro Ser Leu Pro Thr Pro Pro Thr Arg Glu  
1 5 10 15

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 11
- (D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Thr"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Gly Ser Arg Ser Arg Thr Pro Ser Leu Pro Thr Pro Pro Thr Arg Glu  
1 5 10 15

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 6
- (D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Thr"

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 8
- (D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Ser"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Gly Ser Arg Ser Arg Thr Pro Ser Leu Pro Thr Pro Pro Thr Arg Glu  
1 5 10 15

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 6
- (D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Thr"

37

## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 11

(D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Thr"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gly	Ser	Arg	Ser	Arg	Thr	Pro	Ser	Leu	Pro	Thr	Pro	Pro	Thr	Arg	Glu
1				5					10					15	

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 8

(D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Ser"

## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 11

(D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Thr"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gly	Ser	Arg	Ser	Arg	Thr	Pro	Ser	Leu	Pro	Thr	Pro	Pro	Thr	Arg	Glu
1				5					10					15	

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

38

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Lys	Lys	Val	Ala	Val	Val	Arg	Thr	Pro	Pro	Lys	Ser	Pro	Ser	Ser	Ala	Lys
1				5				10					15			

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 8

(D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Thr"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Lys	Lys	Val	Ala	Val	Val	Arg	Thr	Pro	Pro	Lys	Ser	Pro	Ser	Ser	Ala	Lys
1				5				10					15			

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 12

39

(D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Ser"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Lys	Lys	Val	Ala	Val	Val	Arg	Thr	Pro	Pro	Lys	Ser	Pro	Ser	Ser	Ala	Lys
1				5				10						15		

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 8
- (D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Thr"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 12
- (D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Ser"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Lys	Lys	Val	Ala	Val	Val	Arg	Thr	Pro	Pro	Lys	Ser	Pro	Ser	Ser	Ala	Lys
1				5				10						15		

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

40

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ala	Glu	Ile	Val	Tyr	Lys	Ser	Pro	Val	Val	Ser	Gly	Asp	Thr	Ser	Pro	Arg	His	Leu
1					5				10					15				

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 7
- (D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Ser"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Ala	Glu	Ile	Val	Tyr	Lys	Ser	Pro	Val	Val	Ser	Gly	Asp	Thr	Ser	Pro	Arg	His	Leu
1					5				10					15				

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 11
- (D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Ser"

41

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ala	Glu	Ile	Val	Tyr	Lys	Ser	Pro	Val	Val	Ser	Gly	Asp	Thr	Ser	Pro	Arg	His	Leu
1					5				10					15				

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 14

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Thr"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Ala	Glu	Ile	Val	Tyr	Lys	Ser	Pro	Val	Val	Ser	Gly	Asp	Thr	Ser	Pro	Arg	His	Leu
1					5				10					15				

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 15

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Ser"



42

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ala	Glu	Ile	Val	Tyr	Lys	Ser	Pro	Val	Val	Ser	Gly	Asp	Thr	Ser	Pro	Arg	His	Leu
1				5					10					15				

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 7

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Ser"

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 11

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Ser"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ala	Glu	Ile	Val	Tyr	Lys	Ser	Pro	Val	Val	Ser	Gly	Asp	Thr	Ser	Pro	Arg	His	Leu
1				5					10					15				

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

43

## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 7

(D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Ser"

## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 14

(D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Thr"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ala	Glu	Ile	Val	Tyr	Lys	Ser	Pro	Val	Val	Ser	Gly	Asp	Thr	Ser	Pro	Arg	His	Leu
1			5					10						15				

## (2) INFORMATION FOR SEQ ID NO:19:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 7

(D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Ser"

## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 15

(D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Ser"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Ala	Glu	Ile	Val	Tyr	Lys	Ser	Pro	Val	Val	Ser	Gly	Asp	Thr	Ser	Pro	Arg	His	Leu
1			5					10						15				

44

## (2) INFORMATION FOR SEQ ID NO:20:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 11
- (D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Ser"

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 14
- (D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Thr"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Ala Glu Ile Val Tyr Lys Ser Pro Val Val Ser Gly Asp Thr Ser Pro Arg His Leu  
1                      5                      10                      15

## (2) INFORMATION FOR SEQ ID NO:21:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 11
- (D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Ser"

45

## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 15

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Ser"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Ala Glu Ile Val Tyr Lys Ser Pro Val Val Ser Gly Asp Thr Ser Pro Arg His Leu  
1 5 10 15

## (2) INFORMATION FOR SEQ ID NO:22:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 14

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Thr"

## (ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 15

(D) OTHER INFORMATION: /product= "OTHER"

/note= "phosphorylated Ser"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Ala Glu Ile Val Tyr Lys Ser Pro Val Val Ser Gly Asp Thr Ser Pro Arg His Leu  
1 5 10 15

## (2) INFORMATION FOR SEQ ID NO:23:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids

(B) TYPE: amino acid

46

- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 5
- (D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Ser"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 7
- (D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Ser"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Ala	Asp	Glu	Val	Ser	Ala	Ser	Leu	Ala	Lys	Gln	Gly	Leu
1				5				10				

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 6
- (D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Ser"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 11
- (D) OTHER INFORMATION: /product= "OTHER"  
/note= "phosphorylated Ser"

47

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Glu	Glu	Lys	Gly	Lys	Ser	Pro	Val	Pro	Lys	Ser	Pro	Val	Glu	Glu	Lys	Gly
1				5					10					15		

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Ala	Val	Tyr	Thr	Arg	Ile	Met	Met	Asn	Gly	Gly	Arg	Leu	Lys	Arg
1				5					10					15

What is claimed is:

1. A synthetic multiphosphorylated peptide derived from the  $\tau$  protein of the paired helical filaments which is useful as an antigen for preparing an antibody specific for paired helical filaments associated with Alzheimer's disease.
2. The peptide according to claim 1, wherein the peptide contains two phosphorylated amino acids.
3. The peptide according to claim 1, wherein the phosphorylated amino acids are serines and threonines, which are separated by between 1 - 6 amino acids.
4. The peptide according to claim 1, wherein the peptide is derived from a fragment of the  $\tau$  protein consisting of amino acids about 207 to about 222, amino acids about 224 to about 240, and amino acids about 390 to about 408.
5. The peptide according to claim 4, wherein the fragment consists of amino acids 207-222 of  $\tau$  and the peptide is selected from the group consisting of:
  - (a) GRSR(T)P(S)LTPPTRE, SEQ ID NO:5;
  - (b) GRSR(T)PSLP(T)PPTRE, SEQ ID NO:6; and
  - (c) GRSRTP(S)LP(T)PPTRE, SEQ ID NO: 7, wherein the () indicates a phosphorylated amino acid.
6. The peptide according to claim 4, wherein the fragment consists of amino acids 224-240 of  $\tau$  and the peptide is KKVAVVR(T)PPK(S)PSSAK, SEQ ID NO:11, wherein () indicates a phosphorylated amino acid.

7. The peptide according to claim 4, wherein the fragment consists of amino acids 390-408 of  $\tau$  and the peptide is selected from the group consisting of:

- (a) AEIVYK(S)PVV(S)GDTSPRHL, SEQ ID NO:17;
- (b) AEIVYK(S)PVVSGD(T)SPRHL, SEQ ID NO:18;
- (c) AEIVYK(S)PVVSGDT(S)PRHL, SEQ ID NO:19;
- (d) AEIVYKSPVV(S)GD(T)SPRHL, SEQ ID NO:20;
- (e) AEIVYKSPVV(S)GDT(S)PRHL, SEQ ID NO:21;
- (f) AEIVYKSPVVS GD(T)(S)PRHL, SEQ ID NO:22, wherein ()

indicates a phosphorylated amino acid.

8. A method for generating an antibody useful in the specific diagnosis of Alzheimer's disease, comprising the step of:

administering to a mammal a synthetic multiphosphorylated peptide derived from the  $\tau$  protein of the paired helical filaments which is useful as an immunogen for preparing an antibody specific for paired helical filaments associated with Alzheimer's disease.

9. The method according to claim 8 wherein the mammal is a mouse.

10. The method according to claim 8 wherein the antibody is a monoclonal antibody or a fragment thereof.

11. The method according to claim 8 wherein the mammal is a rabbit.

12. The method according to claim 8 wherein the antibody is a polyclonal antibody.



13. The method according to claim 8, wherein the peptide contains two phosphorylated amino acids.

14. The method according to claim 8, wherein the phosphorylated amino acids are serines and threonines which are separated by between 1 and 6 amino acids.

15. The method according to claim 8, wherein the peptide is derived from a fragment of the  $\tau$  protein consisting of amino acids about 207 to about 222, amino acids about 224 to about 240 and amino acids about 390 to about 408.

16. The method according to claim 15, wherein the fragment is amino acids 207-222 of  $\tau$  and the peptide is selected from the group consisting of:

- (a) GSRSR(T)P(S)LTPPTRE, SEQ ID NO:5;
- (b) GSRSR(T)PSLP(T)PPTRE, SEQ ID NO:6; and
- (c) GSRSRTP(S)LP(T)PPTRE, SEQ ID NO:7; wherein the () indicates a phosphorylated amino acid.

17. The method according to claim 15, wherein the fragment is amino acids 224-240 of  $\tau$  and the peptide is peptide is KKVAVVR(T)PPK(S)PSSAK, SEQ ID NO:11, wherein () indicates a phosphorylated amino acid.

18. The method according to claim 15, wherein the fragment is amino acids 390-408 of  $\tau$  and the peptide is selected from the group consisting of:

- (a) AEIVYK(S)PVV(S)GDTSPRHL, SEQ ID NO:17;
- (b) AEIVYK(S)PVVSGD(T)SPRHL, SEQ ID NO:18;
- (c) AEIVYK(S)PVVSGDT(S)PRHL, SEQ ID NO:19;
- (d) AEIVYKSPVV(S)GD(T)SPRHL, SEQ ID NO:20;
- (e) AEIVYKSPVV(S)GDT(S)PRHL, SEQ ID NO:21; and

(f) AEIVYKSPVVSGD(T)(S)PRHL, SEQ ID NO:22; wherein ( ) indicates a phosphorylated amino acid.

19. An antibody generated according to the method of claim 8, which is characterized by for the  $\tau$  protein of the paired helical filaments associated with Alzheimer's disease.

20. An antibody or fragment thereof, which is specific for a synthetic multiphosphorylated peptide according to claim 1, said antibody or antibody fragment selected from the group consisting of:

- (a) a monoclonal antibody;
- (b) a polyclonal antibody;
- (c) a recombinant antibody; and
- (d) Fab, Fv, or F(ab')<sub>2</sub> of any of (a) to (c).

21. The antibody according to claim 20, wherein said recombinant antibody is selected from the group consisting of bifunctional antibodies, chimeric antibodies and humanized antibodies.

22. A method for identifying compounds useful in the treatment and diagnosis of Alzheimer's disease comprising the steps of:

(a) contacting a synthetic multiphosphorylated peptide according to claim 1 with a test compound to permit binding of the test compound to the peptide; and

(b) determining the amount of test compound which is bound to the peptide, wherein specific binding of the compound to the peptide indicates that the test compound is capable of being used for treatment or diagnosis of Alzheimer's disease.

23. A method for identifying compounds useful in the treatment and diagnosis of Alzheimer's disease comprising the steps of:

(a) contacting a synthetic multiphosphorylated peptide according to claim 1 with a known target of said peptide and a test compound to permit binding; and

(b) determining the amount of target which is bound to the peptide, wherein inhibition of binding of target to the peptide indicates binding of the test compound to the peptide, indicating that the test compound is capable of being used for treatment or diagnosis of Alzheimer's disease.

24. The method according to claim 22 or 23, wherein the synthetic multiphosphorylated peptide is immobilized on a solid support.

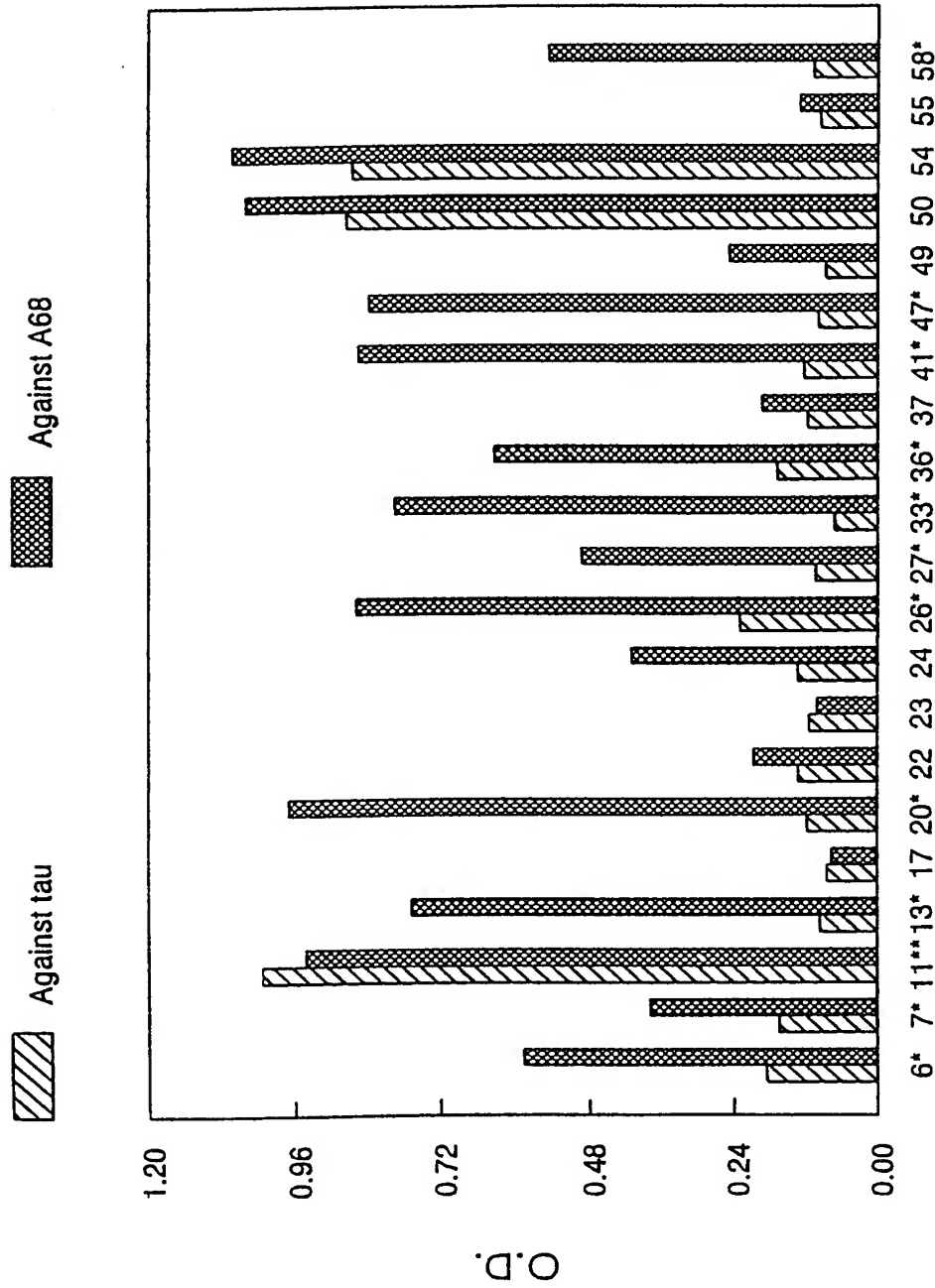
25. A compound identified by the method of any of claims 22 to 24.

26. A method of treating a patient with Alzheimer's disease comprising the step of administering to the patient an antibody or fragment thereof according to any of claims 19 to 21.

27. A method of treating a patient with Alzheimer's disease comprising the step of administering to the patient a multiphosphorylated peptide according to claim 1.

28. Use of an antibody according to any of claims 8 or 19 to 21 in the preparation of a medicament for treatment of Alzheimer's disease.

29. Use of a multiphosphorylated peptide according to claim 1 in the preparation of a medicament for treatment of Alzheimer's disease.



Monoclonal antibodies

FIG.1

**PHF-1**  
1 2 3 4 5 6



At At' Fc Fc' PHF<sub>1</sub> PHF<sub>1</sub>'

**FIG. 2A**

**PHF-6**  
1 2 3 4 5 6



At At' Fc Fc' PHF<sub>6</sub> PHF<sub>6</sub>'

**FIG. 2B**

**PHF-13**  
1 2 3 4 5 6



At At' Fc Fc' PHF<sub>13</sub> PHF<sub>13</sub>'

**FIG. 2C**

**PHF-27**  
1 2 3 4 5 6



At At' Fc Fc' PHF<sub>27</sub> PHF<sub>27</sub>'

**FIG. 2D**

3/15

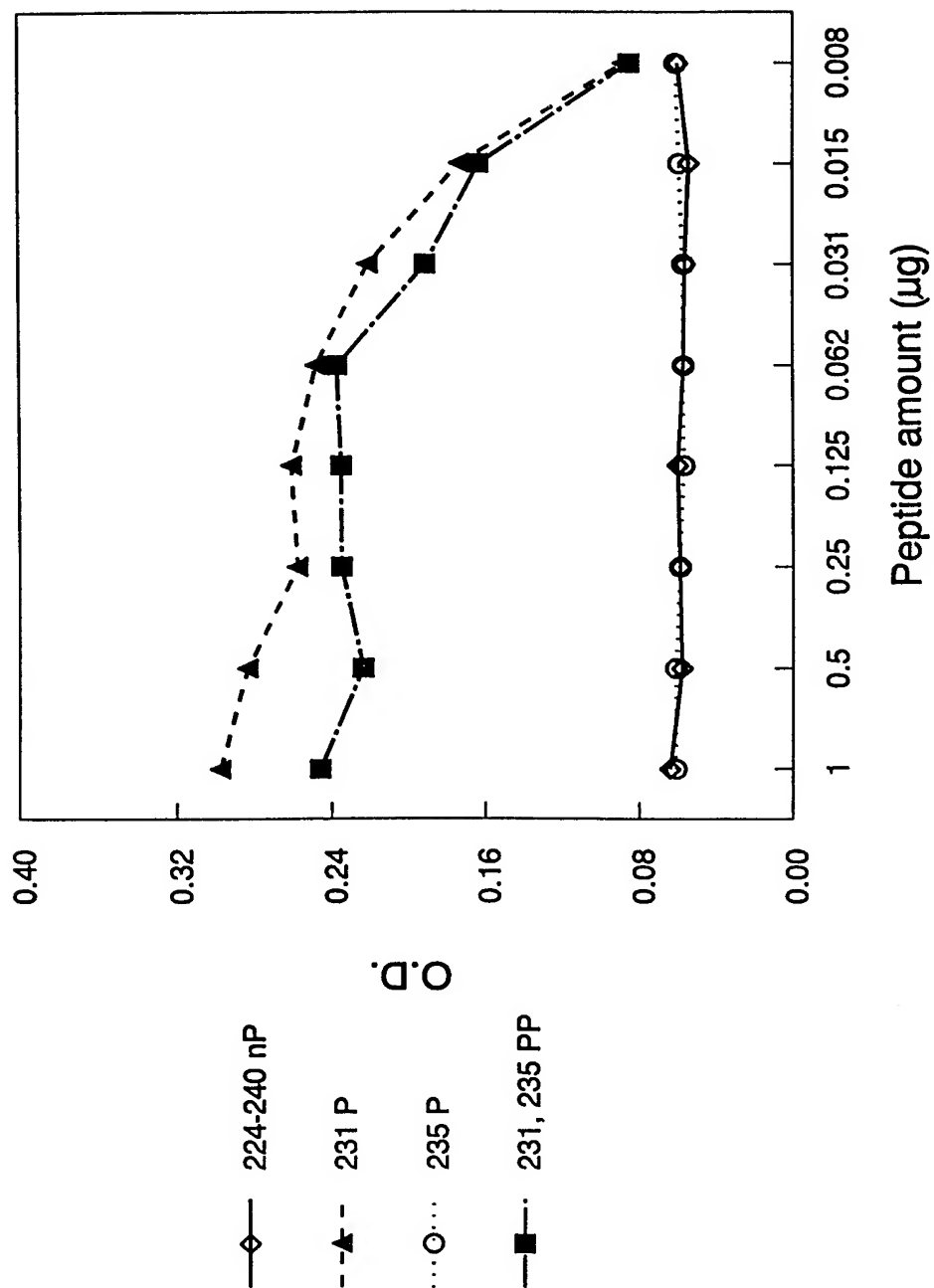


FIG. 3

4/15

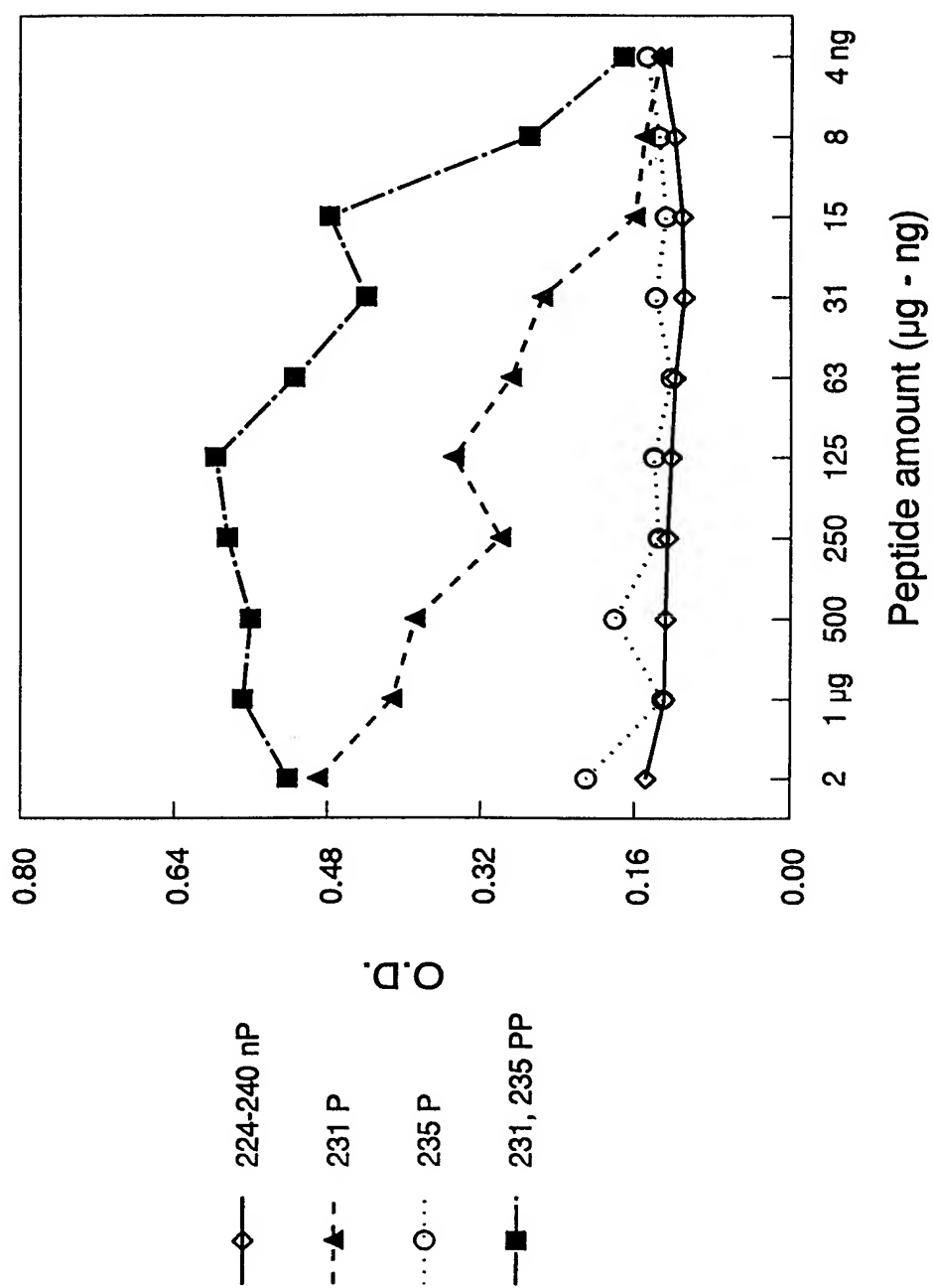


FIG. 4

5/15

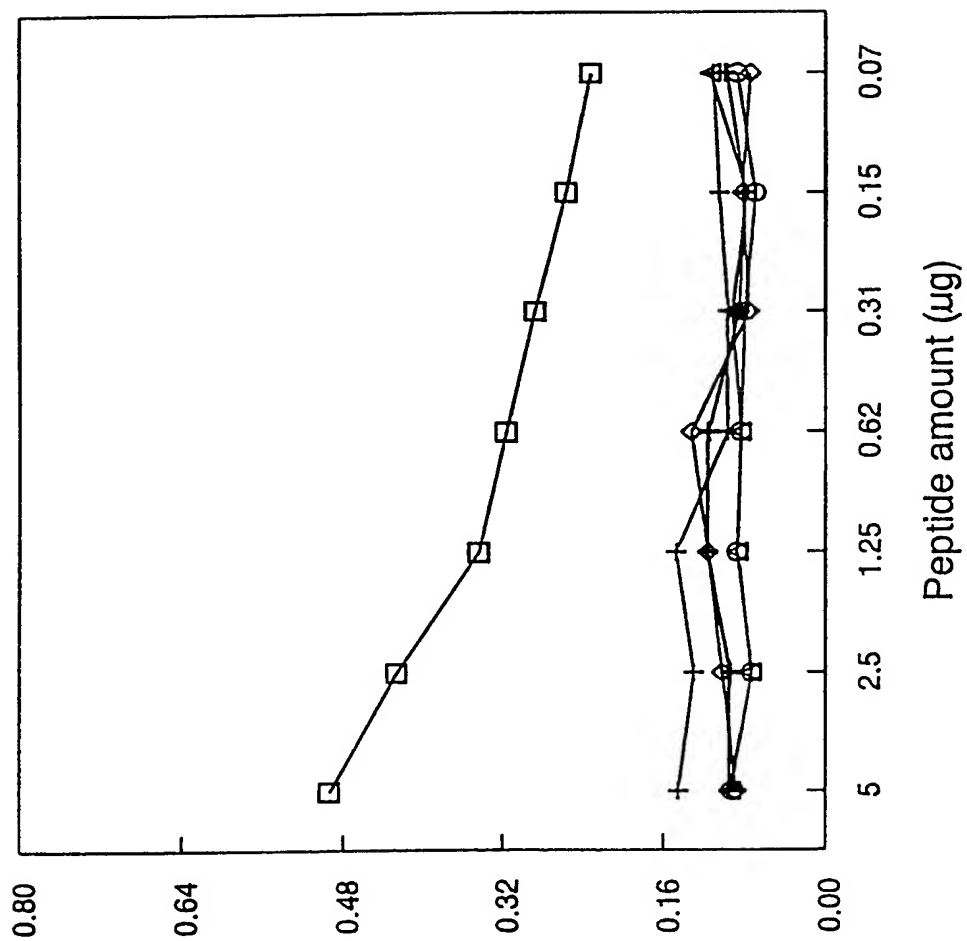


FIG. 5



6/15

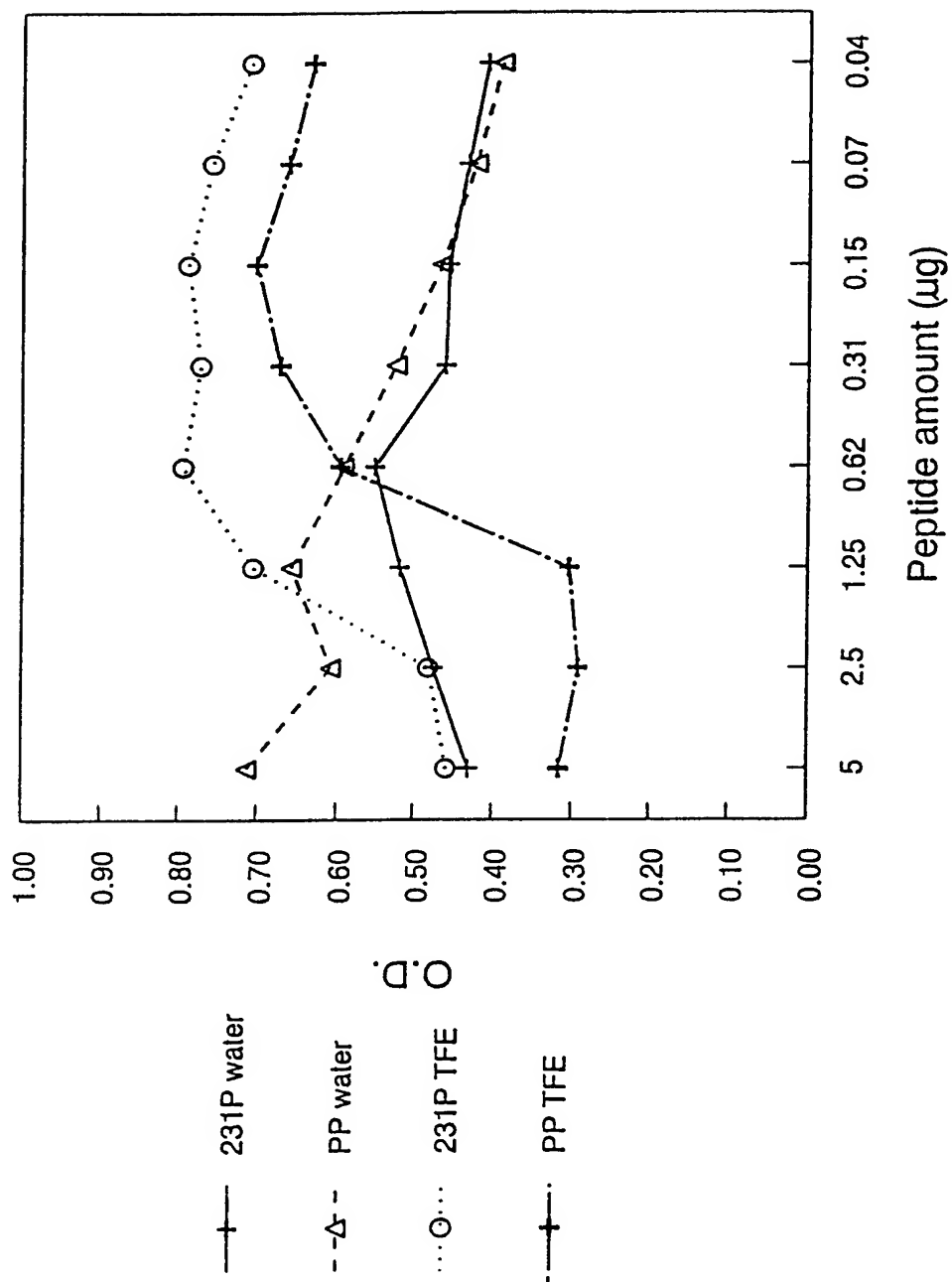


FIG. 6A

7/15

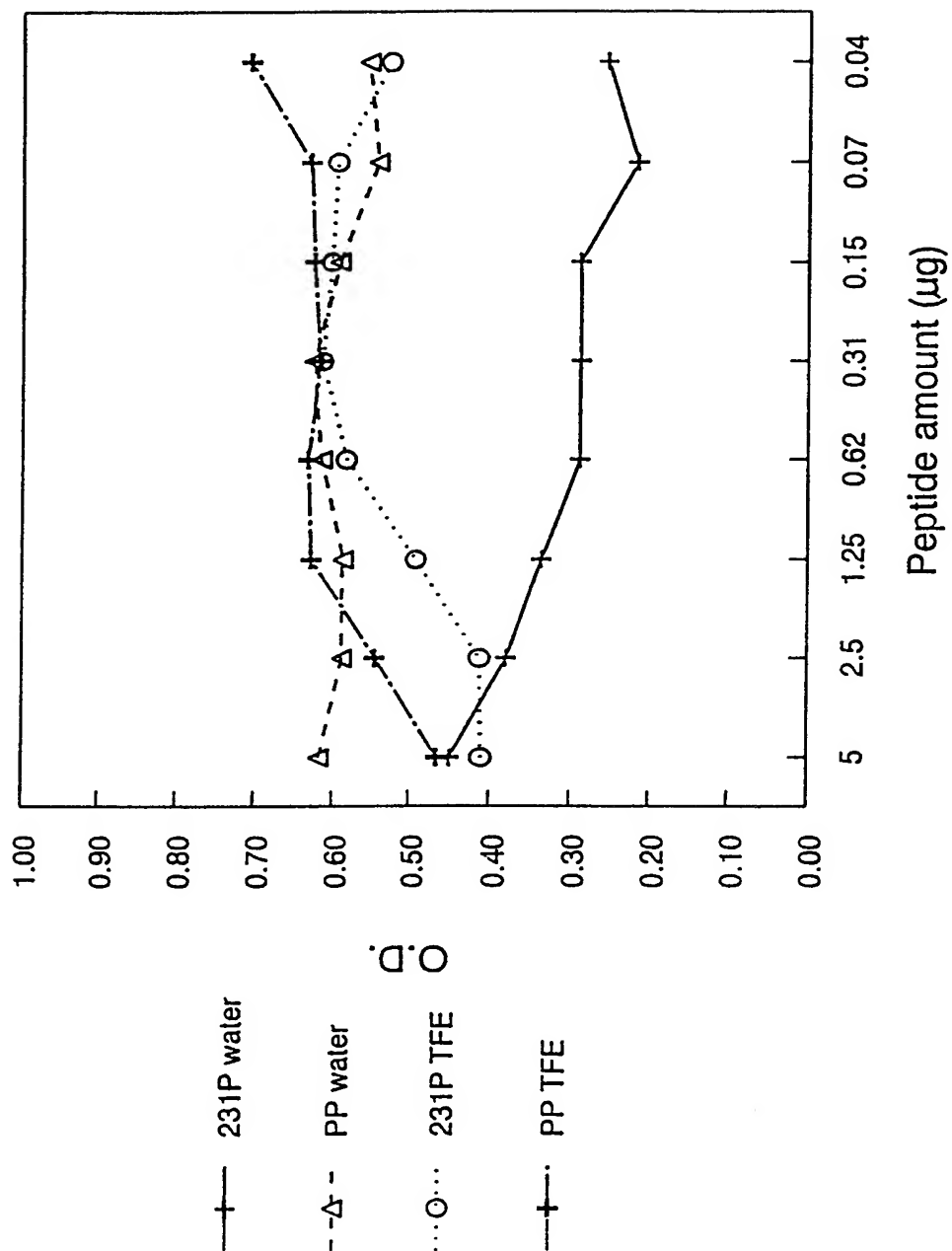


FIG.6B

8/15

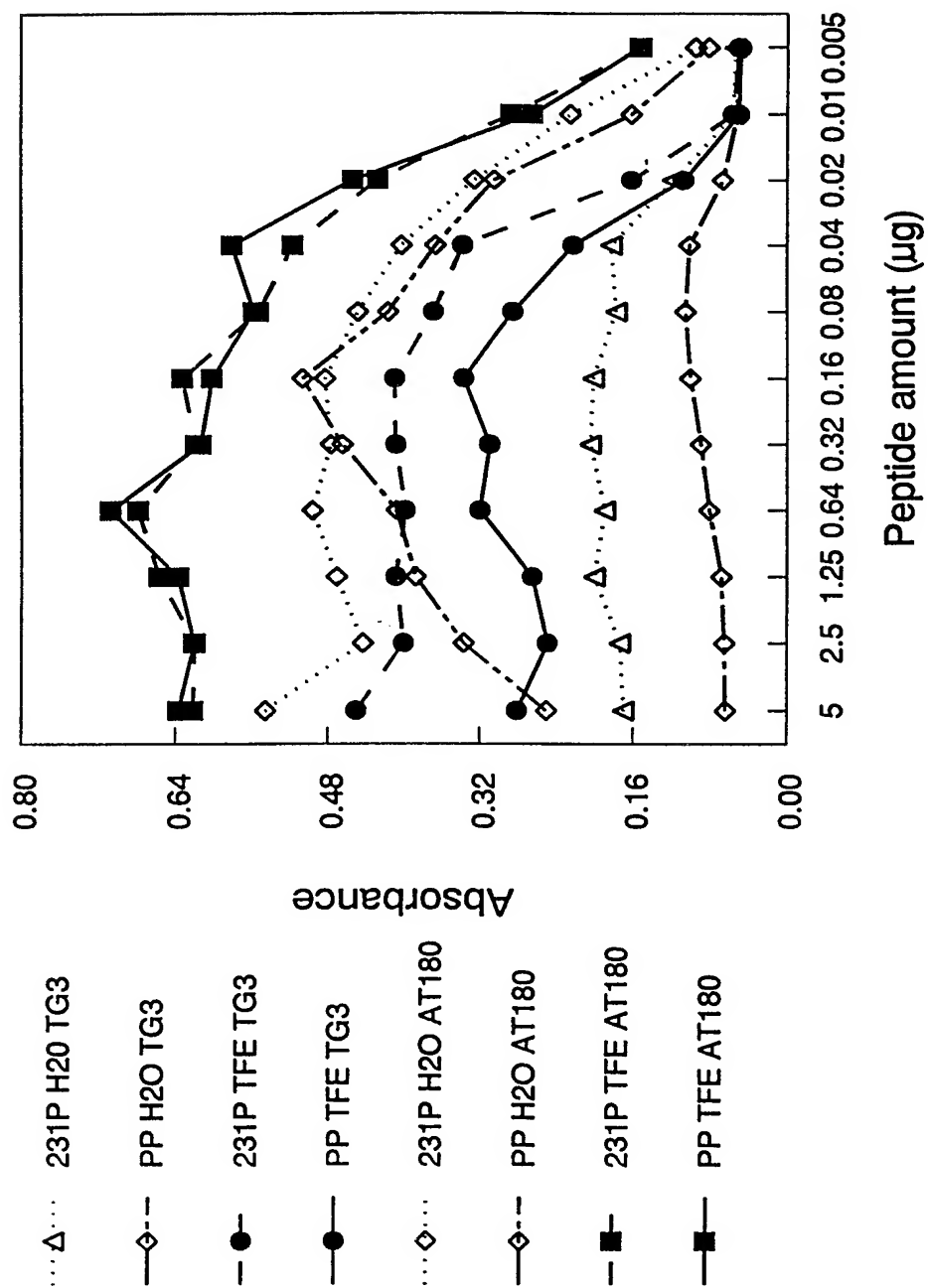


FIG. 7

9/15

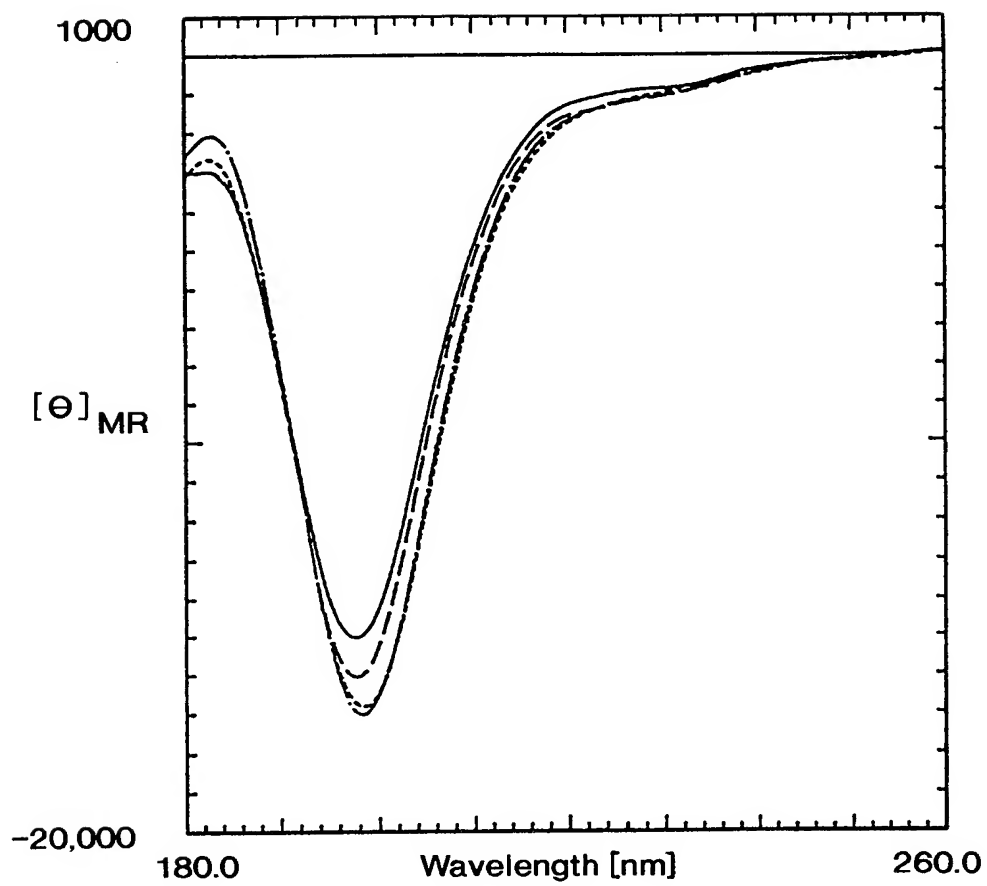


FIG. 8A

10/15

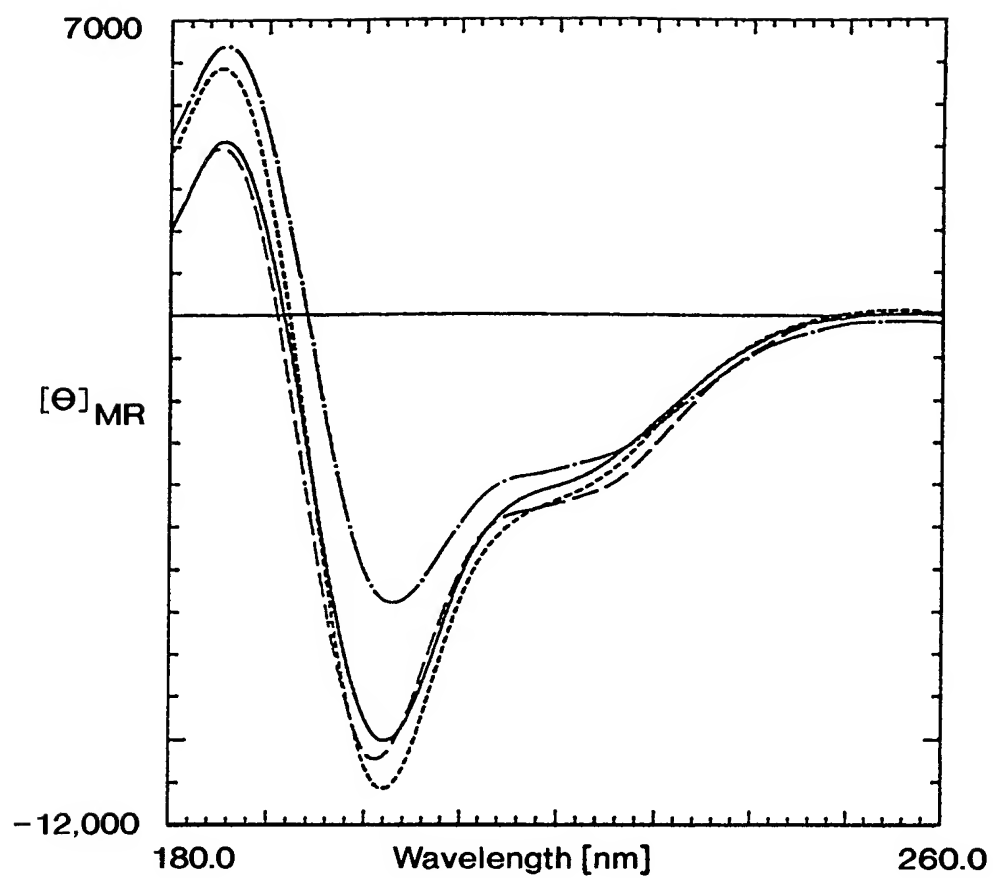


FIG. 8B

11/ 15



FIG. 9A



FIG. 9B

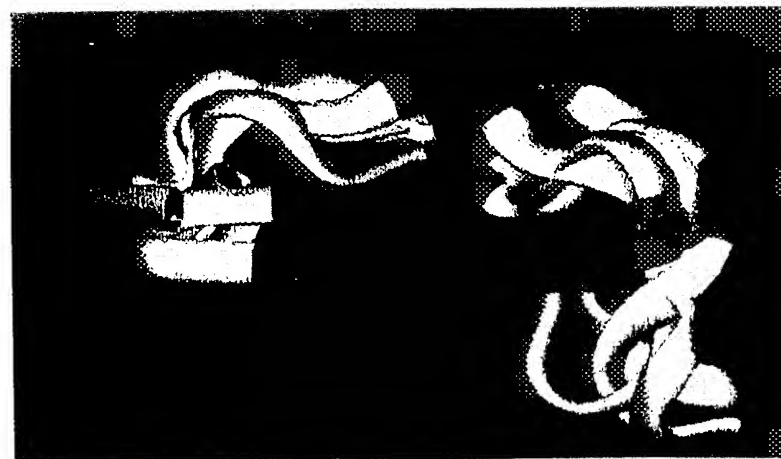


FIG. 9C

12/15

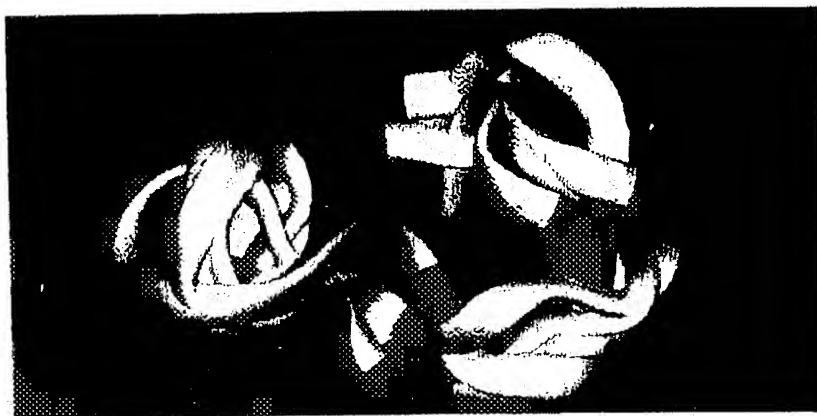


FIG. 9D

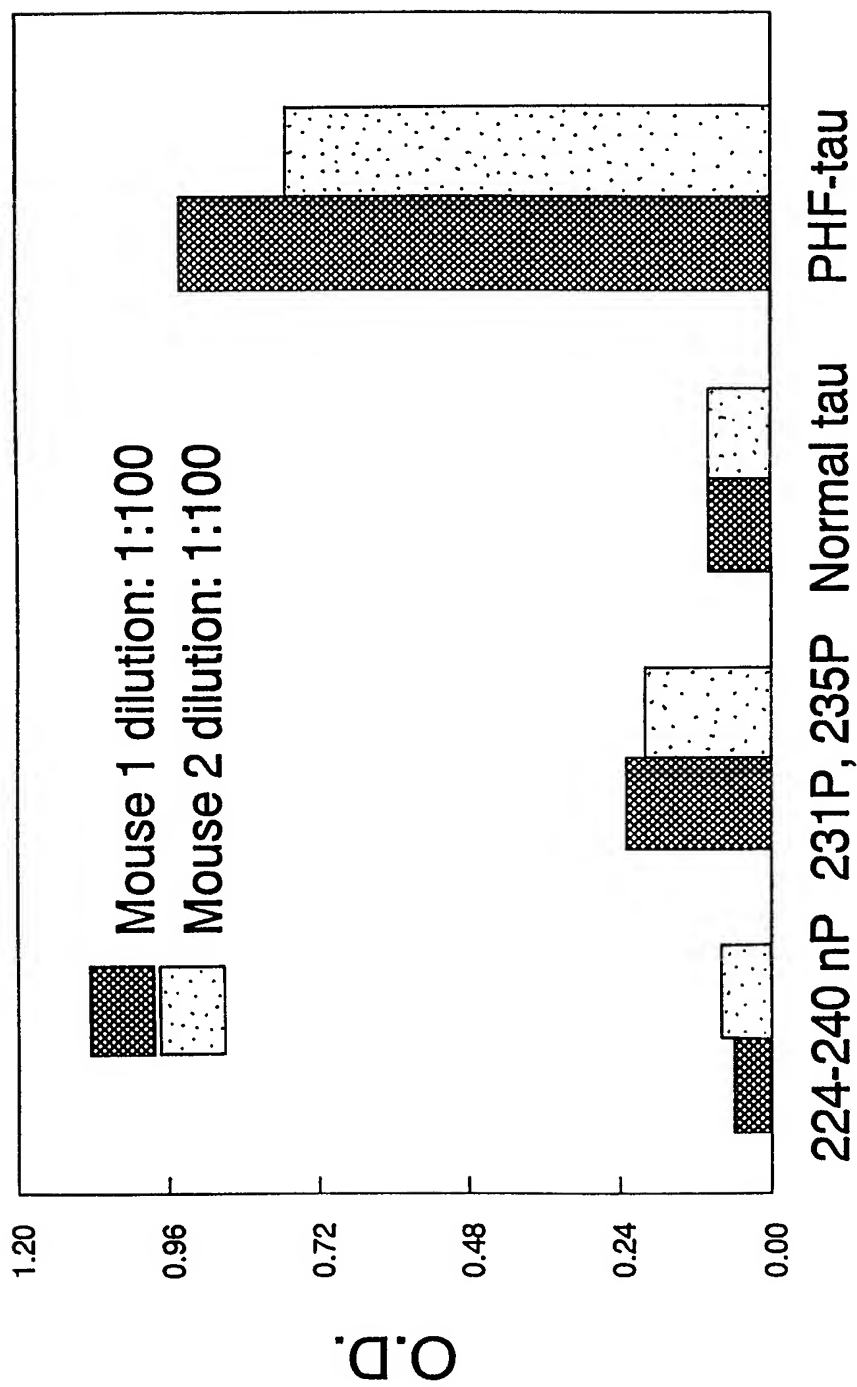


FIG. 9E



FIG. 9F

13/ 15



Antigens  
FIG. 10



14 / 15

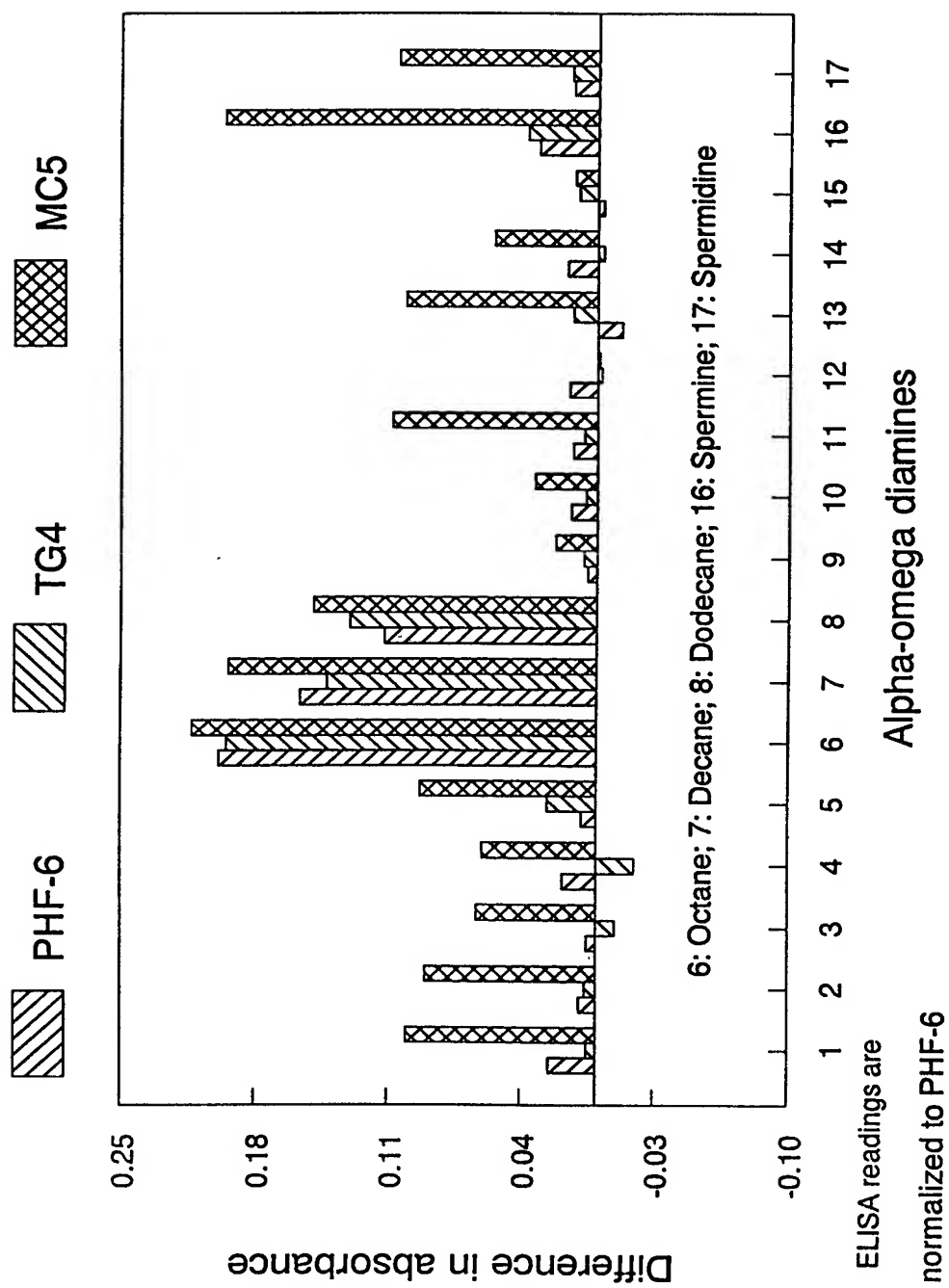
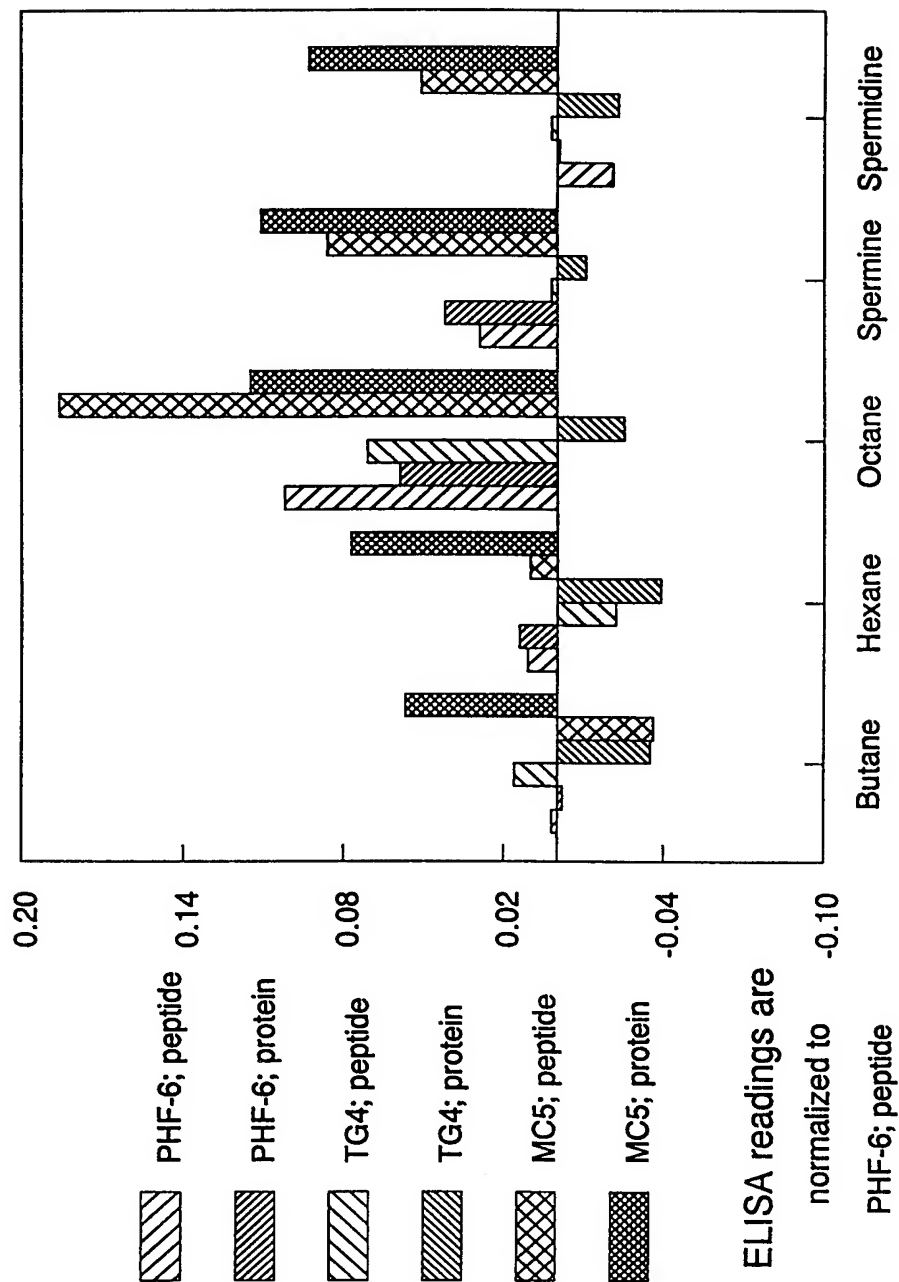


FIG. IIA

PHF-6:231; TG4:235; MC5:both



Alpha-omega diamines

FIG. 11B

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/21116

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet.

US CL : 530/300, 387.1; 435/4

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/300, 387.1; 435/4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG: author and word. Search terms include: tau, phosphory?, antibod?, serine io threonine

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ZEMLAN et al. Monoclonal antibody PHF-9 recognizes phosphorylated serine 404 of tau protein and labels paired helical filaments. Journal of Neuroscience Research. October 1996. Vol. 46, No. 1. pages 90-97, Abstract only.	1-29
Y	SHIURBA et al. Immunohistochemcistry of tau phophoserine 413 and tau protein kinase I in Alzheimer pathology. Brain Research. October 1996. Vol. 737, No. 1-2. pages 119-132, Abstract only.	1-29
Y	Kimura et al. Sequential cahnges of tau-site-specific phosphorylation during development of paired helical filaments. Dememtia. July-August 1996. Vol. 7, No. 4. pages 177-181, Abstract only.	1-29

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

25 FEBRUARY 1998

Date of mailing of the international search report

20 MAR 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

HEATHER BAKALYAR

Telephone No. (703) 308-0196

**INTERNATIONAL SEARCH REPORT**

International application No.  
PCT/US97/21116

**A. CLASSIFICATION OF SUBJECT MATTER:**

IPC (6):

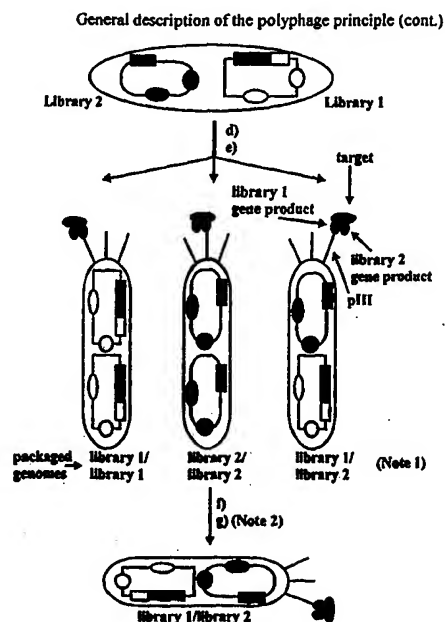
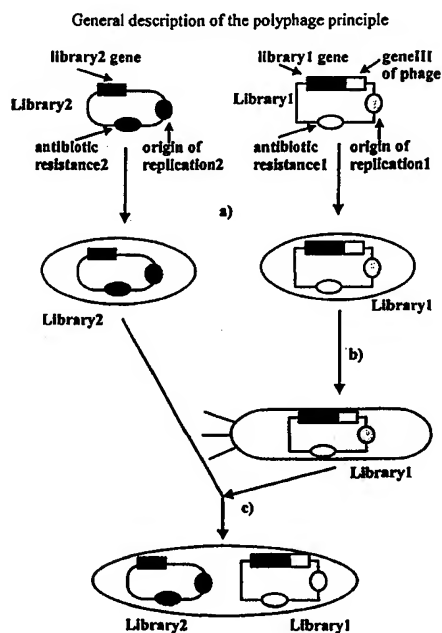
A61K 38/00; C07K 2/00, 4/00, 5/00, 7/00, 14/00, 16/00, 17/00; C12Q 1/00



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12Q</b>	<b>A2</b>	(11) International Publication Number: <b>WO 99/06587</b> (43) International Publication Date: 11 February 1999 (11.02.99)
<p>(21) International Application Number: PCT/EP98/04836</p> <p>(22) International Filing Date: 3 August 1998 (03.08.98)</p> <p>(30) Priority Data: 97113319.4 1 August 1997 (01.08.97) EP</p> <p>(71) Applicant (for all designated States except US): MORPHOSYS GESELLSCHAFT FÜR PROTEINOPTIMIERUNG AG [DE/DE]; Am Klopferspitz 19, D-82152 Martinsried (DE).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): RUDERT, Fritz [DE/DE]; Josef-Retzer-Strasse 36, D-81241 München (DE). GE, Liming [CN/DE]; Portiastrasse 12, D-81545 München (DE). ILAG, Vic [PH/DE]; Knorrstrasse 85, D-89897 München (DE).</p> <p>(74) Agent: VOSSIUS &amp; PARTNER; Siebertstrasse 4, D-81675 München (DE).</p>		<p>(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p><b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i></p>

(54) Title: NOVEL METHOD AND PHAGE FOR THE IDENTIFICATION OF NUCLEIC ACID SEQUENCES ENCODING MEMBERS OF A MULTIMERIC (POLY)PEPTIDE COMPLEX



## (57) Abstract

The present invention relates to methods for the identification of nucleic acid sequences encoding members of a multimeric (poly)peptide complex by screening for polyphage particles. Furthermore, the invention relates to products and uses thereof for the identification of nucleic acid sequences in accordance with the present invention.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## **NOVEL METHOD AND PHAGE FOR THE IDENTIFICATION OF NUCLEIC ACID SEQUENCES ENCODING MEMBERS OF A MULTIMERIC (POLY)PEPTIDE COMPLEX**

The present invention relates to methods for the identification of nucleic acid sequences encoding members of a multimeric (poly)peptide complex by screening for polyphage particles. Furthermore, the invention relates to products and uses thereof for the identification of nucleic acid sequences in accordance with the present invention.

Since its first conception by Ladner in 1988 (WO88/06630), the principle of displaying repertoires of proteins on the surface of phage has experienced a dramatic progress and has resulted in substantial achievements. Initially proposed as display of single-chain Fv (scFv) fragments, the method has been expanded to the display of bovine pancreatic trypsin inhibitor (BPTI) (WO90/02809), human growth hormone (WO92/09690), and of various other proteins including the display of multimeric proteins such as Fab fragments (WO91/17271; WO92/01047).

A Fab fragment consists of a light chain comprising a variable and a constant domain (VL-CL) non-covalently binding to a heavy chain comprising a variable and constant domain (VH-CH1). In Fab display one of the chains is fused to a phage coat protein, and thereby displayed on the phage surface, and the second is expressed in free form, and on contact of both chains, the Fab assembles on the phage surface.

Various formats have been developed to construct and screen Fab phage-display libraries. In its simplest form, just one repertoire, e. g. of heavy chains, is encoded on the phage or phagemid vector. A corresponding light chain, or a repertoire of light chains, is expressed separately. The Fab fragments assemble either inside a host cell, if the light chain is co-expressed from a plasmid, or outside the cell in the medium, if a collection of secreted phage particles each displaying a heavy chain is contacted with the light chain(s) expressed from a different host cell. By screening such Fab libraries, just the information about the heavy chain encoded on the phage or phagemid vector is retrievable, since that vector is packaged in the phage particle. By reverting the format and displaying a library of light chains, and

assembling Fab fragments by co-expressing or adding one or more of the heavy chains identified in the first round, corresponding light chain-heavy chain pairs can be identified.

To avoid that multi-step procedure, both repertoires may be cloned into one phage or phagemid vector, one chain expressible as a fusion with at least part of a phage coat protein, the second expressible in free form. After selection, the phage particle will contain the sequence information about both chains of the selected Fab fragments. The disadvantage of such a format is that the overall complexity of the library is limited by transformation efficiency. Therefore, the library size will usually not exceed  $10^{10}$  members.

For various applications, a library size of up to  $10^{14}$  would be advantageous. Therefore, methods of using site-specific recombination, either based on the Cre/lox system (WO92/20791) or on the att $\lambda$  system (WO 95/21914) have been proposed. Therein, two collection of vectors are sequentially introduced into host cells. By providing the appropriate recombination sites on the individual vectors, recombination between the vectors can be achieved by action of an appropriate recombinase or integrase, achieving a combinatorial library, the overall library size being the product of the sizes of the two individual collections. The disadvantages of the Cre/lox system are that the recombination event is not very efficient, it leads to different products and is reversible. The att $\lambda$  system leads to a defined product, however, it creates one very large plasmid which has a negative impact on the production of phages. Furthermore, the action of recombinase or integrase most likely leads to undesired recombination events.

Thus, the technical problem underlying the present invention is to develop a simple, reliable system which enables the simultaneous identification of members of a multimeric (poly)peptide complex, such as the identification of heavy and light chain of a Fab fragment, in phage display systems.

The solution to this technical problem is achieved by providing the embodiments characterized in the claims. Accordingly, the present invention allows to easily create and screen large libraries of multimeric (poly)peptide complexes for properties such as binding to a target, as in the case of screening Fab fragment libraries, or such as enzymatic activity, as in the case of libraries of multimeric enzymes. The technical approach of the present invention, i.e. the retrieval of information about two members of a multimeric (poly)peptide complex



encoded on two different vectors without requiring a recombination event, is neither provided nor suggested by the prior art.

Accordingly, the present invention relates to a method for identifying a combination of nucleic acid sequences encoding two members of a multimeric (poly)peptide complex with a predetermined property, said combination being contained in a combinatorial library of phage particles displaying a multitude of multimeric (poly)peptides complexes, said method being characterized by screening or selecting for polyphage particles that contain said combination.

Surprisingly, it has been achieved by the present invention that the phenomenon of polyphages can be used to co-package the genetic information of two or more members of multimeric (poly)peptide complexes in a phage display system. The occurrence of polyphage particles has been observed 30 years ago (Salivar et al., Virology 32 (1967) 41-51), where it was described that approximately 5% of a phage population form particles which are longer than unit length and which contain two or more copies of phage genomic DNA. They occur naturally when a newly forming phage coat encapsulates two or more single-stranded DNA molecules. In specific cases, it has been seen that co-packaging of phage and phagemids or single-stranded plasmid vectors takes place as well (Russel and Model, J. Virol. 63 (1989) 3284-3295). Despite of occasional scientific articles about the morphogenesis of polyphage particles, a practical application has never been discussed or even been mentioned. In WO92/20791 in example 26, a model experiment for a combinatorial Fab display library expressed from separate vectors is presented. However, there is only a screening process for either of the two vectors described. Thus, the prior art teaches away from screening for the simultaneous presence of two vectors in a polyphage particle.

In the context of the present invention, the term "multimeric (poly)peptide complex" refers to a situation where two or more (poly)peptide(s) or protein(s), the "members" of said multimeric complex, can interact to form a complex. The interaction between the individual members will usually be non-covalent, but may be covalent, when post-translational modification such as the formation of disulphide-bonds between any two members occurs. Examples for "multimeric (poly)peptide complexes" comprise structures such as fragments derived from immunoglobulins (e. g. Fv, disulphide-linked Fv (dsFv), Fab fragments), fragments derived from other members of the immunoglobulin superfamily (e.g.  $\alpha$ , $\beta$ -

heterodimer of the T-cell receptor), and fragments derived from homo-or heterodimeric receptors or enzymes. In phage display, one of said members is fused to at least part of a phage coat protein, whereby that member is displayed on, and assembly of the multimeric complex takes place at, the phage surface. A "combinatorial phage library" is produced by randomizing at least two members of said multimeric (poly)peptide complex at least partially on the genetic level to create two libraries of genetically diverse nucleic acid sequences in appropriate vectors, by combining the libraries in appropriate host cells and by achieving co-expression of said at least two libraries in a way that a library of phage particles is produced wherein each particle displays one of the possible combinations out of the two libraries.

By screening such a combinatorial phage library displaying multimeric (poly)peptide complexes for a predetermined property, a collection of phage particles will be identified. Partially, these particles will just contain the genetic information of one of the members of the multimeric complex. The inventive principle of the present invention is the screening step for polyphage particles containing the genetic information of a combination of library members.

Furthermore, the present invention relates to a method for identifying a combination of nucleic acid sequences encoding two members of a multimeric (poly)peptide complex with a predetermined property, said combination being contained in a combinatorial library of phage particles displaying a multitude of multimeric (poly)peptides complexes, comprising the steps of

- (a) providing a first library of recombinant vector molecules containing genetically diverse nucleic acid sequences comprising a variety of nucleic acid sequences, each encoding a fusion protein of a first member of a multimeric (poly)peptide complex fused to at least part of a phage coat protein, said fusion protein thereby being able to be directed to, and displayed at, the phage surface, wherein said vector molecules are able to be packaged in a phage particle and carry or encode a first selectable and/or screenable property;
- (b) providing a second library of recombinant vector molecules containing genetically diverse nucleic acid sequences comprising a variety of nucleic acid sequences, each encoding a second member of a multimeric (poly)peptide complex, wherein the vector molecules of said second library are able to be packaged in a phage particle and carry

or encode a second selectable and/or screenable property different from said first property;

- (c) optionally, providing nucleic acid sequences encoding further members of a multimeric (poly)peptide complex;
- (d) expressing members of said libraries of recombinant vectors mentioned in steps (a), (b), and optionally nucleic acid sequences mentioned in step (c), in appropriate host cells under appropriate conditions, so that a combinatorial library of phage particles each displaying a multimeric (poly)peptide complex is produced;
- (e) identifying in said library of phage particles a collection of phages displaying multimeric (poly)peptide complexes having said predetermined property;
- (f) identifying in said collection polyphage particles simultaneously containing recombinant vector molecules encoding a first and a second member of said multimeric (poly)peptide complex by screening or selecting for the simultaneous presence or generation of said first and second selectable and/or screenable property;
- (g) optionally, carrying out further screening and/or selection steps or repeating steps (a) to (f);
- (h) identifying said combination of nucleic acid sequences.

Optionally, further members of said multimeric complex may be provided in the case of ternary, quaternary or higher (poly)peptide complexes. These further members may, for example, be co-expressed from one of the phage or phagemid vectors or from a separate vector such as a plasmid. Even libraries of such further members could be employed in which case further screenable or selectable properties would have to be introduced on the corresponding vectors. Alternatively, such further libraries could be contained in said first or second libraries of recombinant vector molecules. In another option, further screening and/or selection steps or a repetition of the individual steps can be carried out, to optimize the result of obtaining and identifying said nucleic acid sequences.

Furthermore, the present invention relates to a method for identifying a combination of nucleic acid sequences encoding two members of a multimeric (poly)peptide complex with a predetermined property, said combination being contained in a combinatorial library of phage particles displaying a multitude of multimeric (poly)peptides complexes, comprising the steps of

- (a) expressing in appropriate host cells under appropriate conditions

- (aa) genetically diverse nucleic acid sequences contained in a first library of recombinant vector molecules, said nucleic acid sequences comprising a variety of nucleic acid sequences, each encoding a fusion protein of a first member of a multimeric (poly)peptide complex fused to at least part of a phage coat protein, said fusion protein thereby being able to be directed to and displayed at the phage surface, wherein said vector molecules are able to be packaged in a phage particle and carry or encode a first selectable and/or screenable property;
- (ab) genetically diverse nucleic acid sequences contained in a second library of recombinant vector molecules, said nucleic acid sequences comprising a variety of nucleic acid sequences, each encoding a second member of a multimeric (poly)peptide complex, wherein the vector molecules are able to be packaged in a phage particle and carry or encode a second selectable and/or screenable property different from said first property;
- (ac) optionally, nucleic acid sequences encoding further members of a multimeric (poly)peptide complex,  
so that a combinatorial library of phage particles each displaying a multimeric (poly)peptide complex is produced;
- (b) identifying in said library of phage particles a collection of phages displaying multimeric (poly)peptide complexes having said predetermined property;
- (c) identifying in said collection polyphage particles simultaneously containing recombinant vector molecules encoding a first and a second member of said multimeric (poly)peptide complex by screening or selecting for the simultaneous presence or generation of said first and second selectable and/or screenable property;
- (d) optionally, carrying out further screening and/or selection steps or repeating steps (a) to (c);
- (e) identifying said combination of nucleic acid sequences.

In a preferred embodiment of the method of the present invention, the vectors of said first and said second library are a combination of a phage vector and a phagemid vector.

In a further preferred embodiment of the method of the present invention, the vectors of said first and said second library are a combination of two phagemid vectors, said appropriate conditions comprising complementation of phage genes by a helper phage.

In a most preferred embodiment of the method of the present invention said two phagemid vectors are compatible.

The term "compatibility" refers to a property of two phagemids to be able to coexist in a host cell. Incompatibility is connected to the presence of incompatible plasmid origins of replication belonging to the same incompatibility group. An example for compatible plasmid origins of replication is the high-copy number origin ColE1 and the low-copy number origin p15A.

Therefore, in a further preferred embodiment of the method of the present invention, said two phagemid vectors comprise a ColE1 and a p15A plasmid origin of replication.

In a most preferred embodiment of the method of the present invention, said two phagemid vectors comprise a ColE1 and a mutated ColE1 origin.

It could be shown, that two phagemids both having a ColE1-derived plasmid origin of replication can coexist in a cell as long as one of the ColE1 origins carries a mutation.

Particularly preferred is a method, wherein said vectors and/or said helper phage comprise different phage origins of replication.

Most preferred is an embodiment of the method of the present invention, wherein said phage vector, said phagemid vector(s) and/or said helper phage are interference resistant.

The term "interference" refers to a property that phagemids inhibit the production of progeny phage particles by interfering with the replication of the DNA of the phage. "Interference resistance" is a property which overcomes this problem. It has been found that mutations in the intergenic region and/or in gene II contribute to interference resistance (Enea and Zinder, Virology 122 (1982), 222-226; Russel et al., Gene 45 (1986) 333-338). It was identified that phages called IR1 and IR2 (Enea and Zinder, Virology 122 (1982), 222-226), and mutants derived therefrom such as R176 (Russel and Model, J. Bacteriol. 154 (1983) 1064-1076), R382, R407 and R408 (Russel et al., Gene 45 (1986) 333-338) and R383 (Russel and Model, J. Virol. 63 (1989) 3284-3295) are interference resistant by carrying mutations in the untranslated region upstream of gene II and in the gene II coding region.

Therefore, in a preferred embodiment of the method of the present invention, said phage vector, said phagemid vector(s) and/or said helper phage have mutations in the phage intergenic region(s), preferably in positions corresponding to position 5986 of f1, and/or in gene II, preferably in positions corresponding to position 143 of f1.

In a most preferred embodiment said phage vector, said phagemid vector(s) and/or said helper phage are, or are derived from, IR1 mutants such as R176, R382, R383, R407, R408, or from IR2 mutants.

In a further embodiment of the method of the invention, said vectors and/or said helper phage comprise hybrid nucleic acid sequences of f1, fd, and/or M13 derived sequences.

In the context of the present invention, the term "hybrid nucleic sequences" refers to vector elements which comprise sequences originating from different phage(mid) vectors.

Surprisingly, it has been found that a vector constructed combining a part derived from fd phage and a second part derived from R408, a derivative of f1 phages, is interference resistant and additionally, gives predominantly polyphage particles.

Therefore, a most preferred embodiment of the method of the present invention relates to a vector which is, or is derived from, fpep3\_1B-IR3seq with the sequence listed in Figure 4.

In a yet further preferred embodiment of the method according to the present invention, said derivative is a phage comprising essentially the phage origin or replication from fpep3\_1B-IR3seq, the gene II from fpep3\_1B-IR3seq, or a combination of said phage origin of replication and said gene II.

The invention relates in an additional preferred embodiment to a method, wherein said derivative is a phagemid comprising essentially the phage origin or replication from fpep3\_1B-IR3seq, the gene II from fpep3\_1B-IR3seq, or a combination of said phage origin of replication and said gene II.

The invention relates in a further preferred embodiment to a method, wherein said derivative is a helper phage comprising essentially the phage origin or replication from fpep3\_1B-

IR3seq, the gene II from fpep3\_1B-IR3seq, or a combination of said phage origin of replication and said gene II.

Most preferred is an embodiment of the method of the invention, wherein said derivatives comprise the combined fd/f1 origin including the mutation G5737>A (2976 in fpep3\_1B-IR3seq), and/or the mutations G343>A (3989) in gII, and G601>T (4247) in gII/X.

The formation of polyphage particles has been examined in more detail by different groups. It was found that amber mutations in genes VII and IX lead to the amplified production of infectious polyphage particles (Lopez and Webster, Virology 127 (1983) 177-193). A couple of mutants in gene VII (R68, R100) and in gene IX (N18) were identified and further characterized.

Accordingly, in a preferred embodiment of the method of the present invention, the gene VII contained in any of said vectors contains an amber mutation, and most preferably, said mutation is identical to those found in phage vectors R68 or R100.

Further preferred is an embodiment, wherein the gene IX contained in any of said vectors contains an amber mutation, and most preferably said mutation is identical to that found in phage vector N18.

Several phage coat proteins have been used in displaying foreign proteins including the gene III protein (gIIIp), gVIp, and gVIIIp.

In a preferred embodiment of the method of the present invention, said phage coat protein is gIIIp or gVIIIp.

In a particularly preferred embodiment of the method of the present invention, said phage particles are infectious by having a full-length copy of gIIIp.

The gIIIp is a protein comprising three domains. The C-terminal domain is responsible for membrane insertion, the two N-terminal domains are responsible for binding to the F pilus of *E. coli* (N2) and for the infection process (N1).

In a most preferred embodiment of the method of the invention, said phage particles are non-infectious by having no full-length copy of gIIIp, said fusion protein being formed with a truncated version of gIIIp, wherein the infectivity can be restored by interaction of the

displayed multimeric (poly)peptide complexes with a corresponding partner coupled to an infectivity-mediating particle.

In the context of the present invention, the term "infectivity-mediating particle" (IMP) refers to a construct comprising either the N1 domain or the N1-N2 domain. On interaction with a non-infectious phage lacking said domains, infectivity of the phage particles can be restored. The interaction between the non-infectious phage and the IMP can be mediated by a ligand fused to the IMP, which can bind to a partner displayed on the phage. By screening a non-infectious phage display library against a target ligand-IMP construct, restoration of infectivity can be used to select target-binding library members.

In a further preferred embodiment of the method of the invention, said truncated gIIIp comprises the C-terminal domain of gIIIp.

In a yet preferred embodiment of the method of the invention, said truncated gIIIp is derived from phage fCA55.

In addition to the work by Lopey and Webster cited above, Crissman and Smith (Virology 132 (1984) 445-455) could show, that the phage fCA55 which has a large deletion in gene III removing the N-terminal domains and a large part of the C-terminal domain leads exclusively to the formation of polyphages.

Particularly preferred is an embodiment of the method of the invention, wherein said predetermined property is binding to a target.

In a preferred embodiment of the method of the invention, said multimeric (poly)peptide complex is a fragment of an immunoglobulin superfamily member.

In a most preferred embodiment of the method of the invention, said multimeric (poly)peptide complex is a fragment of an immunoglobulin.

In a further most preferred embodiment of the method of the invention, said fragment is an Fv, dsFv or Fab fragment.



An additional preferred embodiment of the present invention relates to a method, wherein said predetermined property is the activity to perform or to catalyze a reaction.

In a preferred embodiment of the method of the invention, said multimeric (poly)peptide complex is an enzyme.

In a most preferred embodiment of the method of the invention, said multimeric (poly)peptide complex is a fragment of a catalytic antibody.

In a further most preferred embodiment of the method of the invention, said fragment is an Fv, dsFv or Fab fragment.

An additional preferred embodiment of the invention relates to a method, wherein selectable and/or screenable property is the transactivation of transcription of a reporter gene such as beta-galactosidase, alkaline phosphatase or nutritional markers such as his3 and leu, or resistance genes giving resistance to an antibiotic such as ampicillin, chloramphenicol, kanamycin, zeocin, neomycin, tetracycline or streptomycin.

In a most preferred embodiment of the method of the invention, said generation of said first and second screenable and/or selectable property is achieved after infection of appropriate host cells by said collection of phage particles.

Particularly preferred is a method, wherein said identification of said nucleic acid sequences is effected by sequencing.

Further preferred is a method, wherein said host cells are E.coli XL-1 Blue, K91 or derivatives, TG1, XL1kann or TOP10F.

An additional preferred embodiment of the invention relates to a polyphage particle which

(a) contains

(i) a first recombinant vector molecule that comprises a nucleic acid sequence, which encodes a fusion protein of a first member of a multimeric (poly)peptide complex

fused to at least part of a phage coat protein, and that carries or encodes a first selectable and/or screenable property, and

(ii) a second recombinant vector molecule that comprises a nucleic acid sequence, which encodes a second member of a multimeric (poly)peptide complex, and that carries or encodes a second selectable and/or screenable property different from said first property;

and (b) displays said multimeric (poly)peptide complex at its surface.

A most preferred embodiment of the invention relates to a polyphage particle, wherein said phage coat protein is the gIIIp.

A further preferred embodiment of the present invention relates to a polyphage particle which is infectious by having a full-length copy of gIIIp present, either in said fusion protein, or in an additional wild-type copy.

Additionally, the invention relates to a polyphage particle which is non-infectious by having no full-length copy of gIIIp, said fusion protein being formed with a truncated version of gIIIp, wherein the infectivity can be restored by interaction of the displayed multimeric (poly)peptide complex with a corresponding partner coupled to an infectivity-mediating particle.

Most preferably, the invention relates to the phage vector fpep3\_1B-IR3seq with the sequence listed in Figure 4.

Additionally preferred, the invention relates to a phage vector derived from phage vector fpep3\_1B-IR3seq comprising essentially the phage origin or replication from fpep3\_1B-IR3seq, the gene II from fpep3\_1B-IR3seq, or a combination of said phage origin of replication and said gene II.

Further preferred is an embodiment of the invention, which relates to a phagemid vector derived from phage vector fpep3\_1B-IR3seq comprising essentially the phage origin or replication from fpep3\_1B-IR3seq, the gene II from fpep3\_1B-IR3seq, or a combination of said phage origin of replication and said gene II.

Preferably, the invention relates to a helper phage vector derived from phage vector fpep3\_1B-IR3seq comprising essentially the phage origin or replication from fpep3\_1B-IR3seq, the gene II from fpep3\_1B-IR3seq, or a combination of said phage origin of replication and said gene II.

Additionally preferred is an embodiment, said derivatives comprise the combined fd/fl origin including the mutation G5737>A (2976 in fpep3\_1B-IR3seq), and/or the mutations G343>A (3989) in gII, and G601>T (4247) in gII/X.

Further preferred is the use of any of the vectors according to the present invention in the generation of polyphage particles containing a combination of at least two different vectors.

Most preferred is the use of vectors of the invention, wherein said combination of different vectors comprises nucleic acid sequences encoding members of a multimeric (poly)peptide complex.

Further preferred in the present invention is the use of vectors, wherein said combination of different vectors comprises nucleic acid sequences encoding interacting (poly)peptides/proteins.

### **Legends-to Figures:**

**Figure 1:** General description of the polyphage principle for the display of a Fab library: e.g. library 1: library of VL chains; library 2: VH chains; both libraries on compatible phagemids; in a: libraries are transformed into host cells; in b: library 1 is rescued by a helper phage; in c: libraries are combined by infection; in d: co-expression of heavy and light chains; in e: rescue by helper phages, production of phage particles, assembly of Fab on phage, selection for target; note 1: A certain fraction of the phage particles will be normal unit-length particles containing just one of the two genomes (not shown in Figure 1). Furthermore, polyphage does not discriminate which genomes to package. Therefore, the combinations shown in Figure 1 can arise. To select for

correctly packaged genomes, the subsequent steps are required; in f: infect host cells; in g: select for ability to confer resistance to two antibiotics to infected cells; note 2: only phage that satisfy condition according to g) represent polyphage particles which contain the correct combination of heavy and light chain of binding Fabs (Hetero-polyphage). Unit-length phage as well as polyphage carrying two identical genomes will confer only resistance to one antibiotics.

- Figure 2:** Functional map and sequence of phage vector fhag1A
- Figure 3:** Functional map and sequence of phage vector fjun\_1B
- Figure 4:** Functional map and sequence of phage vector fpep3\_1B-IR3seq
- Figure 5:** Compatibility of various phage and phagemid vectors: co-transformation of different vector pairs and growth in liquid culture (can/amp selection):  
 A. fjun\_1B-R408-IR/pIG10\_pep10; B. fjun\_1B/pIG10\_pep10 (only 1 colonie);  
 C. fpep3\_1B-IR3/pIG10\_pep10; D. fjun\_1B-R408-IR/pOK1Djun; E. fjun\_1B/pOK1Djun: no growth; F. fpep3\_1B-IR3/pOK1Djun;  
 a. fjun\_1B; b. fjun\_1B-R408-IR; c. fpep3\_1B-IR3; d. pIG10\_pep10; e. pOK1Djun
- Figure 6:** co-transformation of positive (pep3/p75ICD combination, lane 9) and negative (jun/p75ICD, lane 10) pairs; lane 1 to 8: SIP transductants
- Figure 7:** Sensitivity of SIP hetero-polyphage system for selection in solution: #SIP hetero-polyphage transductants, transducing units (t.u.)/ml, produced by co-cultures of co-transformants as in Figure 6 mixed at the indicated ratios.
- Figure 8:** PCR to identify phage vector(s) present in SIP polyphage transductants: lane 1 to 6: SIP polyphage transductants; lane A: fpep3\_1B-IR3/pIG10.3-IMPp75 co-transformant; lane B: fjun\_1B-IR3/pIG10.3-IMPp75 co-transformant
- Figure 9:** IR Phage and Phagemid are Co-packaged into Polyphages: 1:  $\Delta$ gIII phage + gIII plasmid; 2: IR phage+ phagemid
- Figure 10:** SIP Information is Co-transduced by Polyphages: a: IMPp75 on phage vector; b: pep10-gIII-CT fusion on phage vector; c: IMPp75 on phagemid vector; d: pep10-gIII-CT fusion on phagemid vector

The examples illustrate the invention

### **Example 1: Selection for polyphage transductants**

In WO92/01047, page 83, a model experiment for a two-vector system is described which uses a phage vector (fd-CAT2-IV) encoding a light chain and a phagemid vector (pHEN1-III) encoding a heavy chain. The phagemid, grown in *E. coli* HB2151, was rescued with fd-CAT2-IV phage, and functional phage(mid)s produced. By infecting TG1 cells and plating on tetracycline (to select for fd-CAT) and ampicillin (to select for pHEN1), the ratio of phage and phagemid being packaged was determined.

By repeating this experiment, but plating on TYE plates with both antibiotics, polyphage transductants transducing both resistances simultaneously can be selected, and the genetic information contained on the phage and phagemid vector can be retrieved.

By replacing the single light and heavy chain in the constructs mentioned above by corresponding repertoires, a library of Fab-displaying phage particles can be produced. By screening that library against an immobilized target, a collection of phage particles can be identified. Polyphage particles contained in that collection can be identified by transducing both resistances as described above.

### **Example 2: Generation and use of an interference-resistant filamentous phage to co-package the genetic information of co-displayed interacting proteins**

#### **Introduction**

The physical connection of randomly combined genetic information is of vital importance in processes such as interactive screening of two libraries of expressed protein members or for co-expression and co-display of protein pairs which are dependent on the interaction with each other for proper function.

#### **2.1.: Construction of a interference resistant filamentous phage:**

##### **2.1.1.: Construction of fjun\_1B:**

##### **- fhag1A (see Figure 2)**

- a. The phage vector f17/9-hag (Krebber *et al.*, 1995, *FEBS Letters* 377, 227-231) is digested with EcoRV and XmnI. The 1.1 kb fragment containing the anti-HAG Ab gene is isolated

by agarose gel electrophoresis and purified with a Qiagen gel extraction kit. This fragment is ligated into a pre-digested pIG10.3 vector (EcoRV-XmnI). Ligated DNA is transformed into DH5a cells and positive clones are verified by restriction analysis. The recombinant clone is called **pIGHag1A**. All cloning described above and subsequently are according to standard protocols (Sambrook *et al.*, 1989, *Molecular Cloning: a Laboratory Manual*, 2<sup>nd</sup> ed.)

- b. The vector f17/9-hag (Krebber *et al.*, 1995) is digested with EcoRV and StuI. The 7.9 kb fragment is isolated and self-ligated to form the vector **fhag2**.
- c. The chloramphenicol resistance gene (CAT) assembled *via* assembly PCR (Ge and Rudolph, *BioTechniques* 22 (1997) 28-29) using the template pACYC (Cardoso and Schwarz, *J. Appl. Bacteriol.* 72 (1992) 289-293) is amplified by the polymerase chain reaction (PCR) with the primers:  
CAT\_BspEI(for): 5' GAATGCTCATCCGGAGTTC  
CAT\_Bsu36I(rev): 5' TTTCAGTGGCCTCAGGCTAGCACCGGCGTTTAAG
- d. The PCR is done following standard protocols (Sambrook *et al.*, 1989). The amplified product is digested with BspEI and Bsu36I then ligated into pre-digested fhag2 vector (BspEI-Bsu36I; 7.2 kb fragment) to form **fhag2C**.
- e. The vector fhag2C is digested with EcoRI and the ends made blunt by filling-in with Klenow fragment. The flushed vector is self-ligated to form vector **fhag2CdelEcoRI**.
- f. pIGHag1A is digested with XbaI and HindIII. The 1.3 kb fragment containing the anti-HAG gene fused with the C-terminal domain of filamentous phage pIII protein is isolated and ligated with a pre-digested fhag2CdelEcoRI phage vector (XbaI-HindIII; 6.4 kb) to create the vector **fhag1A**.

**- fjun\_1B (see Figure 3)**

- a. The DNA encoding the C-terminal domain including the long linker separating it from the amino terminal domain of the filamentous phage pIII (gIII short) is amplified by PCR using pOK1 (Gramatikoff *et al.*, *Nucleic Acids Res.* 22 (1994) 5761-5762) as template with the primers:  
gIII short(for): 5'GCTTCCGGAGAATTCAATGCTGGCGGCGGCTCT3'  
gIII short(rev): 5'CCCCCCCCAAGCTTATCAAGACTCCTTATTACG3'
- b. The PCR is done following standard protocols (Sambrook *et al.*, 1989). The amplified product is digested with EcoRI and HindIII, then ligated into pre-digested fhag1A vector (EcoRI-HindIII) to form the vector **fjun\_1B**.

### 2.1.2.: Construction of *fjun\_1B-R408IR*:

In order to introduce mutations which have been described to confer an interference resistance phenotype (Enea and Zinder, Virology 122 (1982), 222-226) into the non-interference resistant fd phage vector *fjun\_1B* (see Fig.3), a 1.7 kb fragment of helper phage R408 (Stratagene) comprising the region between the unique restriction sites *DraIII* and *BsrGI* was PCR amplified by assembly PCR. Subfragments of the 1.7 kb *DraIII/BsrGI* fragment were amplified from the f1 phage R408 template DNA with primer combinations FR604/FR605 and FR606/FR607 to introduce via the partially complementary primers FR605 and FR606 an additional *gII* mutation found to be present in the recipient construct *fjun\_1B*. Resulting PCR fragments were gel-purified and combined to serve as template in an subsequent assembly PCR with primers FR604 and FR607. PCR conditions were standard, with approx. 25 ng template, 10 pmole of each primer, 250 pmole of each dNTP, 2 mM Mg, 2.5 U Pfu DNA polymerase (Stratagene). Amplification was done for 30 cycles, with 1 min denaturation at 94 C, 1 min annealing at 50°C, 1 min extension at 72°C. The correct-sized 1.7 kb assembly PCR product was gel-purified, digested with *DraIII* and *BsrGI* and cloned into *DraIII/BsrGI*-digested *fjun\_1B*, generating *fjun\_1B-R408IR*.

Primers:      FR604 5' GTTCACGTAGTGGGCCATCG 3'  
                 FR605 5' TGAGAGGTCTAAAAAGGCTATCAGG 3'  
                 FR606 5' TAGCCTTTTGTAGACCTCTCAAAAATAG 3'  
                 FR607 5' CGGTGTACAGACCAGGCGC 3'

### 2.2.: Proof of principle experiments

Despite of the absence of the two originally associated IR mutations, the hybrid phage vector *fjun\_1B-R408IR* (carrying the chloramphenicol acetyltransferase conferring chloramphenicol resistance) could be co-transformed with a phagemid (pOK1delta*jun*, carrying the beta-lactamase gene conferring ampicillin resistance) containing a phage origin of replication. More importantly, *fjun\_1B-R408IR* could stably co-exist with the phagemid pOK1delta*jun*, and the phagemid was efficiently co-packaged together with the *fjun\_1B-R408IR* phage genome into polyphage particles. Titers of polyphages, simultaneously

transducing chloramphenicol and ampicillin resistance, reached  $6 \times 10^8$  transducing units (t.u.)/ml of overnight bacterial culture K91 plating cells, a number almost equivalent to a titer of  $10^9$ /ml seen after selection on chloramphenicol only. Selection of the K91 transductants on ampicillin only gave a titer of  $5 \times 10^9$ /ml. These titers indicated that more than 50 % of all phages containing fjun\_1B-R408IR also contained the phagemid pOK1deltajun, thus representing polyphages. This high ratio of polyphages was confirmed by restriction analysis of transductants which had been selected on chloramphenicol only. More than 50 % of these clones also contained the phagemid in addition to the fjun\_1B-R408IR phage genome. fjun\_1B-R408IR was isolated in pure form from an individual transductant, which contained only this phage. The construct fjun\_1B-R408IR was used with pOK1deltajun for co-transformation of DH5 $\alpha$  cells, in order to produce selectively-infective phages (SIP) via fos-jun leucine zipper interaction (which non-covalently restores wt gIII function). Stable, double-resistant co-transformants were obtained with this combination and individual clones were grown overnight in the presence of cam/amp. The culture supernatant of these clones was filtered through a 45  $\mu$ M membrane filter and used to infect exponentially-growing F<sup>+</sup> bacteria (K91 strain) for 20 min at 37 C. To test for the presence of infective SIP polyphages the cells were plated on LB agar plates containing cam and amp and plates were incubated at 37 C overnight. Approx. 500 to 1000 transforming units (t.u.)/ml resulting in double-resistant transductants were obtained from individual co-transformants. DNA of those transductants was analyzed by restriction analysis which showed that 95 % (15/16 clones) of the clones had the correct pattern expected for fjun\_1B-R408IR and pOK1deltajun. Supernatants of several polyphage transductants were tested for persistent SIP phage production by re-infection of K91 cells. This confirmed that polyphage transductants continued to produce infective SIP phages and restriction analysis of the resulting 2<sup>nd</sup> round polyphage transductants showed that 44 % (14/32 clones) contained the correct vector combination. The rest of the clones contained the correct pOK1deltajun phagemid plus a recombined phage vector with a restored wt gIII, indicating an increase in recombination frequency when both vectors are propagated in the rec<sup>+</sup> strain K91 (compared to the rec<sup>-</sup> strain DH5 $\alpha$  used for co-transformation of IR phage and phagemid). To test other protein-protein interactions which give a higher titer of infective SIP phages and to verify the presence of hetero-polyphages (co-packaging of phage and phagemid instead of co-infection by monophages or homo-polyphages) , two peptide ligands (previously selected by SIP, WO97/32017)



which bind to the p75 rat neurotrophin receptor (Chao et al., Science 232 (1986) 518-521) intracellular domain (p75ICD) were cloned as N-terminal gIIc fusions in fjun\_1B-R408IR (replacing jun) and the phagemid pIG10.3, leading to constructs fpep3\_1B-IR3seq and pIG10.3-pep10 (WO97/32017), respectively, which contain the peptide pep3: 5'-TGTATTGTTTATCATGCTCATTATCTTGTTGCTAAGTGT-3' encoding the amino acid sequence (CysIleValTyrHisAlaHisTyrLeuValAlaLysCys) instead of the jun sequence. Sequencing of the respective parts of the transferred R408 fragment in fpep3\_1B-IR3seq revealed that neither of the two IR mutations (the G5986>A mutation from complementation group I in the gII 5' non-translated region, which should be found at position 3225 in fpep3\_1B-IR3seq, and the C143>T mutation (3789 in fpep3\_1B-IR3seq) from complementation group II leading to a Thr>Ile amino acid exchange in gII) were found to be present. However, the gII mutation G6090>T (3329 in fpep3\_1B-IR3seq), leading to a Leu>Val exchange, introduced by assembly PCR was present. Furthermore, three additional mutations compared to an f1 phage could be identified: G5737>A (2976 in fpep3\_1B-IR3seq) in the phage origin of replication, G343>A (3989) in gII, and G601>T (4247) in gII/X.

The functional map and the sequence of fpep3\_1B-IR3seq are given in Figure 4. This sequence was double-checked several times. It could be shown that differences in the sequence of fpep3\_1B-IR3seq compared to published sequence data could be explained by mutations already present in the starting constructs used for cloning fjun\_1B-R408IR and fpep3\_1B-IR3seq.

Co-transformation experiments (Fig. 5) using combinations of pIG10.3 or pOK1 phagemids (both with f1 oris) with fjun\_1B ("wt" fd phage), fjun\_1B-R408-IR (containing the DraIII/BsrGI fragment from R408) or fpep3\_1B-IR3 (containing the DraIII/BsrGI fragment from R408 and the PCR mutation) revealed that the PCR mutation is not necessary for the IR phenotype, at least judged by the ability to be co-transformable with a phagemid and the ability of individual co-transformants to grow in liquid culture (cam/amp selection).

Additionally, the interacting protein partner p75ICD was cloned as a C-terminal fusion to the infectivity-mediating domains (N1-N2) of gIII (infectivity-mediating particle (IMP) fusion) resulting in constructs fIMPp75-IR3 and pIG10.3-IMPp75.

The IR phage was tested with the SIP pairing fpep3\_1B-IR3seq3/ pIG10.3-IMPp75 (which gives a higher titer than fos/jun SIP) in the presence of the negative control combination fjun\_1B-IR3seq3/ pIG10.3-IMPp75 (Fig. 6). A SIP hetero-polyphage titer of  $1.5 \times 10^5$ /ml (cam/amp-resistant transductants) was achieved with fpep3\_1B-IR3seq3/ pIG10.3-IMPp75. To test SIP sensitivity in a model library vs. library setting, co-transformants of fpep3\_1B-IR3seq3/ pIG10.3-IMPp75 were diluted in an excess fjun\_1B-IR3/ pIG10.3-IMPp75 and the supernatant of the bacterial co-culture was assayed for SIP hetero-polyphages. This showed that down to a dilution of  $10^{-5}$  to  $10^{-6}$  can be recovered (Fig. 7).

To prove that only the correct phage vector is present in SIP polyphage transductants, DNA of positive (fpep3\_1B-IR3seq3/ pIG10.3-IMPp75) and negative (fjun\_1B-IR3/ pIG10.3-IMPp75) control co-transformants, as well as DNA from the SIP polyphage transductants derived from SIP phages produced by the mix of positive and negative control bacteria was analyzed by PCR (Fig. 8). Primers FR614 (5'-GCTCTAGATAACGAGGGC-3') and FR627 (5'-CGCAAGCTTAAGACTCCT-TATTACGC-3') amplify the phage region from the start of ompA to the end of gIII. PCR products derived from fpep3\_1B-IR3seq3 and fjun\_1B-IR3 can be discriminated by size. Gel analysis of the above samples verified that only the expected fpep3\_1B-IR3seq3 phage was present in SIP polyphage transductants (6 analyzed).

To physically demonstrate the existence of hetero-polyphages (which have phage and phagemid co-packaged) when using the IR phage vector, phages produced by co-transformants of fIR3/pIG10.3-IMPp75 and as a control fjun\_1B/JB61 ("wt" phage plus complementing gIII plasmid) were separated on an agarose gel (Fig. 9). This showed that the fIR3/pIG10.3-IMPp75 combination produced substantially more slower migrating (thus bigger) phages than the fjun\_1B/JB61 control combination. The ratio was almost inversed. Elution of phages from various regions of the gel and subsequent titering of the eluate on plating cells showed that the upper gel region contained a significant portion of double resistance-transducing phages which thus can be regarded as hetero-polyphages.

The pairs fpep3\_1B-IR3 and pIG10.3-IMPp75 as well as fIMPp75-IR3 and pIG10.3-pep10 were co-transformed into DH5 $\alpha$ , individual cam/amp resistant clones were grown and the culture supernatant was tested on K91 cells for SIP phage production (Fig. 10). The combinations fpep3\_1B-IR3/pIG10.3-IMPp75 and fIMPp75-IR3/pIG10.3-pep10 gave a titer of  $1.5 \times 10^5$  t.u./ml and  $5 \times 10^3$  t.u./ml, respectively when assayed for cam/amp-resistant transductants. The titer for each combination when assayed on LB cam was nearly the same as when assayed on LB cam/amp. This demonstrated efficient co-packaging of phage and phagemid DNA to almost 100 %, as seen before with the initial fjun\_1B-R408IR and pOK1deltajun combination. To proof the existence of polyphages which individually co-transduce phage and phagemid DNA simultaneously, and to rule out the possibility of transduction of the two resistance markers by independent (and thus random) co-infection by two different phages which have only phage or phagemid packaged, a statistical test was performed. Defined, identical aliquots of bacterial culture supernatants of an individual co-transformant representing each of the two SIP vector combinations described above (fpep3\_1B-IR3/pIG10.3-IMPp75 and fIMPp75-IR3/pIG10.3-pep10) were either used individually to infect K91 cells followed by selection on LB cam and LB amp plates, or the same supernatant aliquots from the two vector combinations were mixed before infection of K91 cells and selection on LB cam/amp. 117 cam-resistant, 328 amp-resistant and 141 cam/amp-resistant transforming units were present in the supernatant aliquot from the fIMPp75-IR3/pIG10.3-pep10 combination and 40 cam-resistant, 30 amp-resistant and 23 cam/amp-resistant transforming units were present in the supernatant aliquot from the fpep3\_1B-IR3/pIG10.3-IMPp75 combination. The mix of both supernatant aliquots contained 166 cam-resistant and 162 cam/amp-resistant transforming units, exactly corresponding to the expected numbers which would be obtained by adding up the transducing units of the two individual aliquots. 48 cam/amp-resistant transductant colonies were picked from the plate where the mix of the two individual aliquots was used for infection and were analyzed by restriction digest. This showed that only the correct, SIP phage-producing vector combination (5 clones containing the fpep3\_1B-IR3/pIG10.3-IMPp75 and 43 clones containing the fIMPp75-IR3/pIG10.3-pep10 combination; this represents a ratio of the two input vector combinations in the analyzed transductants of 1 : 8.6 (fpep3\_1B-IR3/pIG10.3-IMPp75 : fIMPp75-IR3/pIG10.3-pep10), which is very similar to the 1 : 6.1 (fpep3\_1B-IR3/pIG10.3-IMPp75 : fIMPp75-IR3/pIG10.3-pep10) ratio of double-resistant input phages in this experiment) occurred in all analyzed

transductants, verifying the presence of hetero-polyphages by ruling out the possibility of random co-infection and thus incorrect, random combination by two out of four possible monophage and/or homo-polyphage populations (fpep3\_1B-IR3, pIG10.3-IMPp75, fIMPp75-IR3 and pIG10.3-pep10) each containing only one type of vector (phage or phagemid). Statistically, co-infection of the same bacterium by two separate phages was practically already excluded by the small numbers of infective phages containing at least one resistance marker (166 cam-resistant and 358 amp-resistant phages) which were used in the above experiment. Co-infection of the same bacterium (of a total of  $10^7$  bacteria) by one of the 166 cam-resistant phages and one of the 358 amp-resistant phages has a probability of  $6 \times 10^{-10}$ . Moreover, in this scenario incorrect combinations of individual phage and phagemid vectors (e.g. fpep3\_1B-IR3/ pIG10.3-pep10 and fIMPp75-IR3/ pIG10.3-IMPp75) would be possible. The fact that only the correct vector combinations were found in all 48 transductants analyzed from this experiment further proved that co-transduction by hetero-polyphage and not random co-infection by homo-polyphage or monophage was the mechanism by which double-resistance was transduced.

### **2.3.: Construction of a phage-display system for Fab display**

The constructs described in 3.2. can easily be modified to achieve the display of Fabs or a Fab library. In fpep3\_1B-IR3seq, the jun part can be replaced by a VL-CL light chain repertoire having the appropriate 3'- and 5'-restriction sites similarly as described for pep\_3-to construct fVL\_1B-R408IR. In pIG10.3-IMPp75, the IMPp75 construct can be replaced by a repertoire of VH-CH1 heavy chains. After co-transformation of both repertoires into host cells and expression, a library of phage particles displaying Fab fragments is produced. Since fpep3\_1B-IR3seq was set up for a SIP experiment by having just the C-terminal domain of gIII, the corresponding Fab-displaying phage particles are non-infectious. By adding a target molecule fused to an infectivity-mediating particle (N1-N2 domain of gIIIp), phages displaying target-binding Fab fragments can be selected by infecting host cells.

By replacing the truncated gIII part described above by a full-length copy of gIII, a Fab-display library of infectious phage particles is obtained, which can be screened against immobilized targets. Binding phages can be eluted and used to infect host cells.

By selecting for transductants conferring cam/amp-resistance to their host cells, polyphage infections can be selected in both cases. Thereby the information about both chains of the selected Fab fragments can be retrieved.

## CLAIMS

1. A method for identifying a combination of nucleic acid sequences encoding two members of a multimeric (poly)peptide complex with a predetermined property, said combination being contained in a combinatorial library of phage particles displaying a multitude of multimeric (poly)peptides complexes,  
said method being characterized by screening or selecting for polyphage particles that contain said combination.
2. The method of claim 1, comprising the steps of
  - (a) providing a first library of recombinant vector molecules containing genetically diverse nucleic acid sequences comprising a variety of nucleic acid sequences, each encoding a fusion protein of a first member of a multimeric (poly)peptide complex fused to at least part of a phage coat protein, said fusion protein thereby being able to be directed to, and displayed at, the phage surface, wherein said vector molecules are able to be packaged in a phage particle and carry or encode a first selectable and/or screenable property;
  - (b) providing a second library of recombinant vector molecules containing genetically diverse nucleic acid sequences comprising a variety of nucleic acid sequences, each encoding a second member of a multimeric (poly)peptide complex, wherein the vector molecules of said second library are able to be packaged in a phage particle and carry or encode a second selectable and/or screenable property different from said first property;
  - (c) optionally, providing nucleic acid sequences encoding further members of a multimeric (poly)peptide complex;
  - (d) expressing members of said libraries of recombinant vectors mentioned in steps (a), (b), and optionally nucleic acid sequences mentioned in step (c), in appropriate host cells under appropriate conditions, so that a combinatorial library of phage particles each displaying a multimeric (poly)peptide complex is produced;
  - (e) identifying in said library of phage particles a collection of phages displaying multimeric (poly)peptide complexes having said predetermined property;
  - (f) identifying in said collection polyphage particles simultaneously containing recombinant vector molecules encoding a first and a second member of said

- multimeric (poly)peptide complex by screening or selecting for the simultaneous presence or generation of said first and second selectable and/or screenable property;
- (g) optionally, carrying out further screening and/or selection steps or repeating steps (a) to (f);
- (h) identifying said combination of nucleic acid sequences.

3. The method of claim 1, comprising the steps of

- (a) expressing in appropriate host cells under appropriate conditions
- (aa) genetically diverse nucleic acid sequences contained in a first library of recombinant vector molecules, said nucleic acid sequences comprising a variety of nucleic acid sequences, each encoding a fusion protein of a first member of a multimeric (poly)peptide complex fused to at least part of a phage coat protein, said fusion protein thereby being able to be directed to and displayed at the phage surface, wherein said vector molecules are able to be packaged in a phage particle and carry or encode a first selectable and/or screenable property;
- (ab) genetically diverse nucleic acid sequences contained in a second library of recombinant vector molecules, said nucleic acid sequences comprising a variety of nucleic acid sequences, each encoding a second member of a multimeric (poly)peptide complex, wherein the vector molecules are able to be packaged in a phage particle and carry or encode a second selectable and/or screenable property different from said first property;
- (ac) optionally, nucleic acid sequences encoding further members of a multimeric (poly)peptide complex,
- so that a combinatorial library of phage particles each displaying a multimeric (poly)peptide complex is produced;
- (b) identifying in said library of phage particles a collection of phages displaying multimeric (poly)peptide complexes having said predetermined property;
- (c) identifying in said collection polyphage particles simultaneously containing recombinant vector molecules encoding a first and a second member of said multimeric (poly)peptide complex by screening or selecting for the simultaneous presence or generation of said first and second selectable and/or screenable property;

- (d) optionally, carrying out further screening and/or selection steps or repeating steps (a) to (c);
- (e) identifying said combination of nucleic acid sequences.
4. The method of anyone of claims 1 to 3, wherein the vectors of said first and said second library are a combination of a phage vector and a phagemid vector.
  5. The method of anyone of claims 1 to 3, wherein the vectors of said first and said second library are a combination of two phagemid vectors, said appropriate conditions comprising complementation of phage genes by a helper phage.
  6. The method of claim 5, wherein said two phagemid vectors are compatible.
  7. The method of claim 6, wherein said two phagemid vectors comprise a ColE1 and a p15A plasmid origin of replication.
  8. The method of claim 6, wherein said two phagemid vectors comprise a ColE1 and a mutated ColE1 origin.
  9. The method of anyone of claims 4 to 8, wherein said vectors and/or said helper phage comprise different phage origins of replication.
  10. The method of anyone of claim 4 to 9, wherein said phage vector, said phagemid vector(s) and/or said helper phage are interference resistant.
  11. The method of claim 10, wherein said phage vector, said phagemid vector(s) and/or said helper phage have mutations in the phage intergenic region(s), preferably in positions corresponding to position 5986 of f1, and/or in gene II, preferably in positions corresponding to position 143 of f1.
  12. The method of anyone of claims 10 to 11, wherein said phage vector, said phagemid vector(s) and/or said helper phage are, or are derived from, IR1 mutants such as R176, R382, R383, R407, R408, or from IR2 mutants.



13. The method of anyone of claims 4 to 11, wherein said vectors and/or said helper phage comprise hybrid nucleic acid sequences of f1, fd, and/or M13 derived sequences.
14. The method of anyone of claims 1 to 13, wherein said vector is, or is derived from, fpep3\_1B-IR3seq with the sequence listed in Figure 4.
15. The method of claim 14, wherein said derivative is a phage comprising essentially the phage origin or replication from fpep3\_1B-IR3seq, the gene II from fpep3\_1B-IR3seq, or a combination of said phage origin of replication and said gene II.
16. The method of claim 14, wherein said derivative is a phagemid comprising essentially the phage origin of replication from fpep3\_1B-IR3seq, the gene II from fpep3\_1B-IR3seq, or a combination of said phage origin of replication and said gene II.
17. The method of claim 14, wherein said derivative is a helper phage comprising essentially the phage origin of replication from fpep3\_1B-IR3seq, the gene II from fpep3\_1B-IR3seq, or a combination of said phage origin of replication and said gene II.
18. The method of anyone of claims 15 to 17, said derivatives comprise the combined fd/f1 origin including the mutation G5737>A (2976 in fpep3\_1B-IR3seq), and/or the mutations G343>A (3989) in gII, and G601>T (4247) in gII/X.
19. The method of anyone of claims 1 to 18, wherein the gene VII contained in any of said vectors contains an amber mutation.
20. The method of claim 19, wherein said mutation is identical to those found in phage vectors R68 or R100.
21. The method of anyone of claims 1 to 20, wherein the gene IX contained in any of said vectors contains an amber mutation.

22. The method of claim 21, wherein said mutation is identical to that found in phage vector N18.
23. The method of anyone of claims 1 to 22, wherein said phage coat protein is gIIIp or gVIIIp.
24. The method of anyone of claims 1 to 23, wherein said phage particles are infectious by having a full-length copy of gIIIp.
25. The method of anyone of claims 1 to 24, wherein said phage particles are non-infectious by having no full-length copy of gIIIp, said fusion protein being formed with a truncated version of gIIIp, wherein the infectivity can be restored by interaction of the displayed multimeric (poly)peptide complexes with a corresponding partner coupled to an infectivity-mediating particle.
26. The method of claim 25, wherein said truncated gIIIp comprises the C-terminal domain of gIIIp.
27. The method of claim 26, wherein said truncated gIIIp is derived from phage fCA55.
28. The method of anyone of claims 1 to 27, wherein said predetermined property is binding to a target.
29. The method of claim 28, wherein said multimeric (poly)peptide complex is a fragment of an immunoglobulin superfamily member.
30. The method of claim 29, wherein said multimeric (poly)peptide complex is a fragment of an immunoglobulin.
31. The method of claim 30, wherein said fragment is an Fv, dsFv or Fab fragment.
32. The method of anyone of claims 1 to 27, wherein said predetermined property is the activity to perform or to catalyze a reaction.

33. The method of claim 32, wherein said multimeric (poly)peptide complex is an enzyme.
34. The method of claim 33, wherein said multimeric (poly)peptide complex is a fragment of a catalytic antibody.
35. The method of claim 34, wherein said fragment is an Fv, dsFv or Fab fragment.
36. The method of anyone of claims 1 to 35, wherein said selectable and/or screenable property is the transactivation of transcription of a reporter gene such as beta-galactosidase, alkaline phosphatase or nutritional markers such as his3 and leu, or resistance genes giving resistance to an antibiotic such as ampicillin, chloramphenicol, kanamycin, zeocin, neomycin, tetracycline or streptomycin.
37. The method of anyone of claims 1 to 36, wherein said generation of said first and second screenable and/or selectable property is achieved after infection of appropriate host cells by said collection of phage particles.
38. The method of anyone of claims 1 to 37, wherein said identification of said nucleic acid sequences is effected by sequencing.
39. The method of anyone of claims 1 to 38, wherein said host cells are E.coli XL-1 Blue, K91 or derivatives thereof, TG1, XL1kann or TOP10F.
40. A polyphage particle which
  - (a) contains
    - (i) a first recombinant vector molecule that comprises a nucleic acid sequence, which encodes a fusion protein of a first member of a multimeric (poly)peptide complex fused to at least part of a phage coat protein, and that carries or encodes a first selectable and/or screenable property, and
    - (ii) a second recombinant vector molecule that comprises a nucleic acid sequence, which encodes a second member of a multimeric (poly)peptide complex, and that

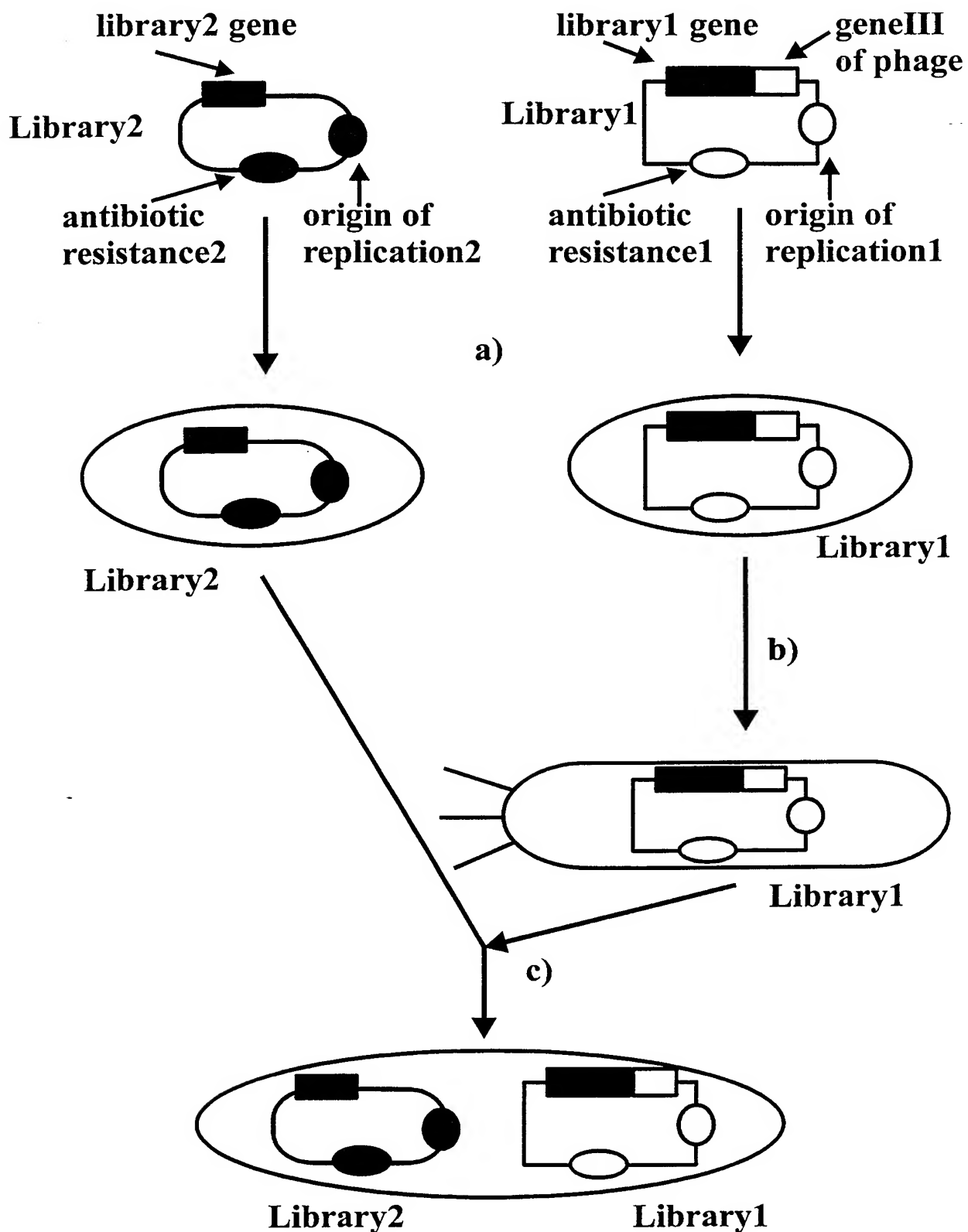
carries or encodes a second selectable and/or screenable property different from said first property;

and (b) displays said multimeric (poly)peptide complex at its surface.

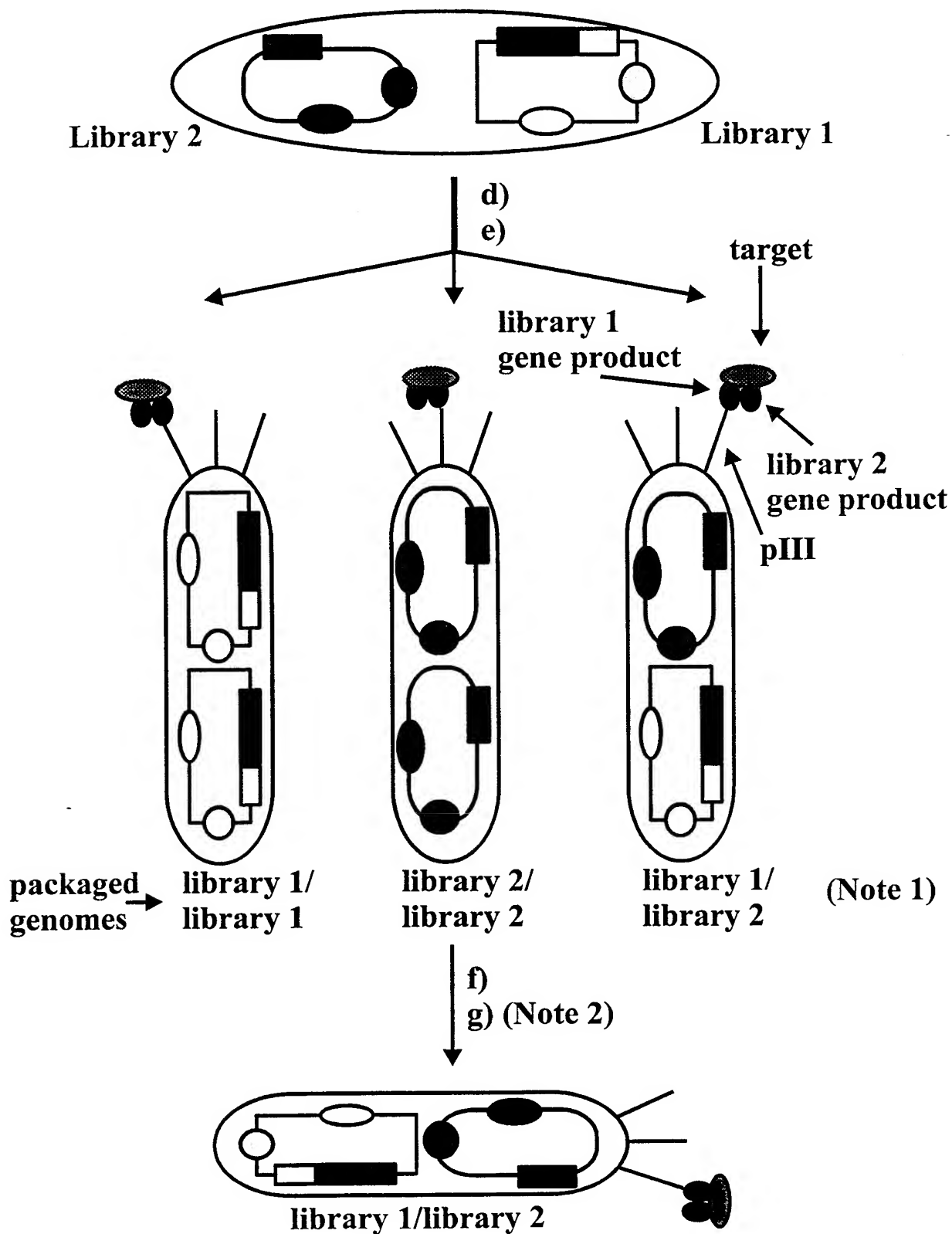
41. The polyphage particle according to claim 40 wherein said phage coat protein is the gIIIp.
42. The polyphage particle according to claim 41 wherein said particles is infectious by having a full-length copy of gIIIp present, either in said fusion protein, or in an additional wild-type copy.
43. The polyphage particle according to claim 41 wherein said particles is non-infectious by having no full-length copy of gIIIp, said fusion protein being formed with a truncated version of gIIIp, wherein the infectivity can be restored by interaction of the displayed multimeric (poly)peptide complex with a corresponding partner coupled to an infectivity-mediating particle.
44. The phage vector fpep3\_1B-IR3seq with the sequence listed in Figure 4.
45. A phage vector derived from phage vector fpep3\_1B-IR3seq comprising essentially the phage origin or replication from fpep3\_1B-IR3seq, the gene II from fpep3\_1B-IR3seq, or a combination of said phage origin of replication and said gene II.
46. A phagemid vector derived from phage vector fpep3\_1B-IR3seq comprising essentially the phage origin or replication from fpep3\_1B-IR3seq, the gene II from fpep3\_1B-IR3seq, or a combination of said phage origin of replication and said gene II.
47. A helper phage vector derived from phage vector fpep3\_1B-IR3seq comprising essentially the phage origin or replication from fpep3\_1B-IR3seq, the gene II from fpep3\_1B-IR3seq, or a combination of said phage origin of replication and said gene II.
48. A vector according to anyone of claims 45 to 47, wherein said derivatives comprise the combined fd/f1 origin including the mutation G5737>A (2976 in fpep3\_1B-IR3seq), and/or the mutations G343>A (3989) in gII, and G601>T (4247) in gII/X.

49. The use according to any of the vectors of anyone of claims 44 to 48 in the generation of polyphage particles containing a combination of at least two different vectors.
50. The use according to claim 49, wherein said combination of different vectors comprises nucleic acid sequences encoding members of a multimeric (poly)peptide complex.
51. The use according to claim 50, wherein said combination of different vectors comprises nucleic acid sequences encoding interacting (poly)peptides/proteins.

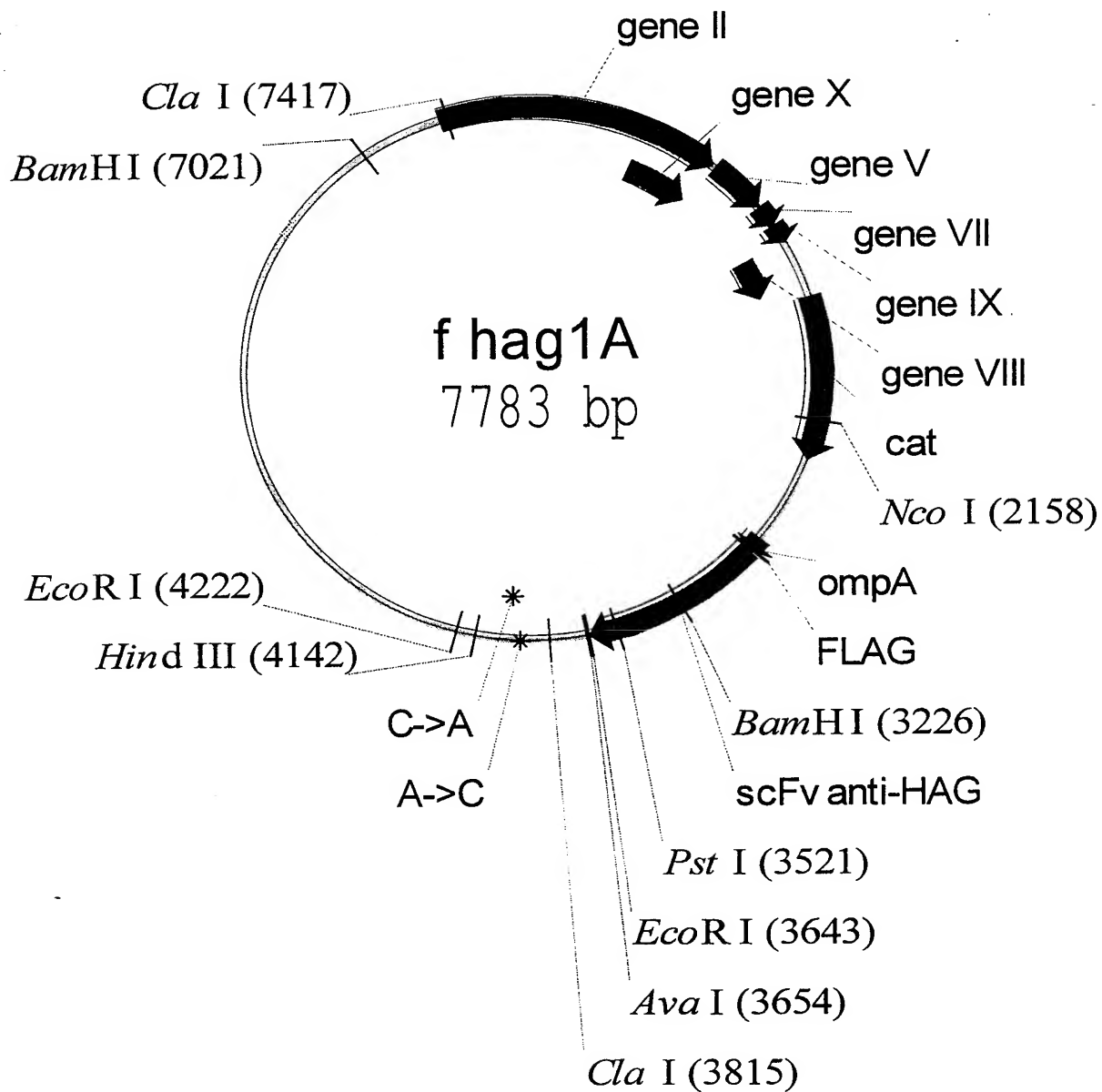
1/39

**Figure 1: General description of the polyphage principle**

2/39

**Figure 1: General description of the polyphage principle (cont.)**

3/39

**Figure 2**



4/39

1	AACGCTACTA	CCATTAGTAG	AATTGATGCC	ACCTTTTTCAG	CTCGCGCCCC
	TTGCGATGAT	GGTAATCATC	TTAACTACGG	TGGAAAAGTC	GAGCGCGGGG
51	AAATGAAAAT	ATAGCTAAAC	AGGTTATTGA	CCATTTGCGA	AATGTATCTA
	TTTACTTTTA	TATCGATTTG	TCCAATAACT	GGTAAACGCT	TTACATAGAT
101	ATGGTCAAAC	TAAATCTACT	CGTTCGCAGA	ATTGGGAATC	AACTGTTACA
	TACCAGTTTG	ATTTAGATGA	GCAAGCGTCT	TAACCCTTAG	TTGACAATGT
151	TGGAATGAAA	CTTCCAGACA	CCGTACTTTA	GTTGCATATT	TAAAACATGT
	ACCTTACTTT	GAAGGTCTGT	GGCATGAAAT	CAACGTATAA	ATTTTGTACA
201	TGAACTACAG	CACCAGATTC	AGCAATTAAG	CTCTAAGCCA	TCCGCAAAAA
	ACTTGATGTC	GTGGTCTAAG	TCGTTAATTC	GAGATTCGGT	AGGCGTTTTT
251	TGACCTCTTA	TCAAAAGGAG	CAATTAAAGG	TACTGTCTAA	TCCTGACCTG
	ACTGGAGAAT	AGTTTTTCCTC	GTTAATTTCC	ATGACAGATT	AGGACTGGAC
301	TTGGAATTTG	CTTCCGGTCT	GGTTCGCTTT	GAGGCTCGAA	TTGAAACGCG
	AACCTTAAAC	GAAGGCCAGA	CCAAGCGAAA	CTCCGAGCTT	AACCTTGCGC
351	ATATTTGAAG	TCTTTCGGGC	TTCCTCTTAA	TCTTTTTGAT	GCAATTCGCT
	TATAAACTTC	AGAAAGCCCG	AAGGAGAATT	AGAAAAACTA	CGTTAAGCGA
401	TTGCTTCTGA	CTATAATAGA	CAGGGTAAAG	ACCTGATTTT	TGATTTATGG
	AACGAAGACT	GATATTATCT	GTCCCATTTT	TGGACTAAAA	ACTAAATACC
451	TCATTCTCGT	TTTCTGAACT	GTTTAAAGCA	TTTGAGGGGG	ATTCAATGAA
	AGTAAGAGCA	AAAGACTTGA	CAAATTTTCGT	AAACTCCCCC	TAAGTTACTT
501	TATTTATGAC	GATTCCGCAG	TATTGGACGC	TATCCAGTCT	AAACATTTTA
	ATAAATACTG	CTAAGGCGTC	ATAACCTGCG	ATAGGTCAGA	TTTGTAAAAA
551	CAATTACCCC	CTCTGGCAAA	ACTTCCTTTG	CAAAAGCCTC	TCGCTATTTT
	GTTAATGGGG	GAGACCGTTT	TGAAGGAAAC	GTTTTCGGAG	AGCGATAAAA
601	GGTTTCTATC	GTCGTCTGGT	TAATGAGGGT	TATGATAGTG	TTGCTCTTAC
	CCAAAGATAG	CAGCAGACCA	ATTACTCCCA	ATACTATCAC	AACGAGAATG
651	CATGCCTCGT	AATTCCTTTT	GGCGTTATGT	ATCTGCATTA	GTTGAGTGTG
	GTACGGAGCA	TTAAGGAAAA	CCGCAATACA	TAGACGTAAT	CAACTCACAC
701	GTATTCCTAA	ATCTCAATTG	ATGAATCTTT	CCACCTGTAA	TAATGTTGTT
	CATAAGGATT	TAGAGTTAAC	TACTTAGAAA	GGTGGACATT	ATTACAACAA
751	CCGTTAGTTC	GTTTTATTAA	CGTAGATTTT	TCCTCCCAAC	GTCCTGACTG
	GGCAATCAAG	CAAATAATT	GCATCTAAAA	AGGAGGGTTG	CAGGACTGAC
801	GTATAATGAG	CCAGTTCTTA	AAATCGCATA	AGGTAATTCA	AAATGATTAA
	CATATTACTC	GGTCAAGAAT	TTTAGCGTAT	TCCATTAAGT	TTTACTAATT

5/39

851	AGTTGAAATT	AAACCGTCTC	AAGCGCAATT	TACTACCCGT	TCTGGTGTTT
	TCAACTTTAA	TTTGGCAGAG	TTCGCGTTAA	ATGATGGGCA	AGACCACAAA
901	CTCGTCAGGG	CAAGCCTTAT	TCACTGAATG	AGCAGCTTTG	TTACGTTGAT
	GAGCAGTCCC	GTTCGGAATA	AGTGACTTAC	TCGTGCAAAC	AATGCAACTA
951	TTGGGTAATG	AATATCCGGT	GCTTGTCAAAG	ATTACTCTCG	ACGAAGGTCA
	AACCCATTAC	TTATAGGCCA	CGAACAGTTC	TAATGAGAGC	TGCTTCCAGT
1001	GCCAGCGTAT	GCGCCTGGTC	TGTACACCGT	GCATCTGTCC	TCGTTCAAAG
	CGGTCGCATA	CGCGGACCAG	ACATGTGGCA	CGTAGACAGG	AGCAAGTTTC
1051	TTGGTCAGTT	CGGTTCTCTT	ATGATTGACC	GTCTGCGCCT	CGTTCCGGCT
	AACCAGTCAA	GCCAAGAGAA	TACTAACTGG	CAGACGCGGA	GCAAGGCCGA
1101	AAGTAACATG	GAGCAGGTCTG	CGGATTTCTGA	CACAATTTAT	CAGGCGATGA
	TTCATTGTAC	CTCGTCCAGC	GCCTAAAGCT	GTGTTAAATA	GTCCGCTACT
1151	TACAAATCTC	CGTTGTACTT	TGTTTCGCGC	TTGGTATAAT	CGCTGGGGGT
	ATGTTTAGAG	GCAACATGAA	ACAAAGCGCG	AACCATATTA	GCGACCCCCA
1201	CAAAGATGAG	TGTTTTAGTG	TATTCTTTCTG	CCTCTTTCTG	TTTAGGTTGG
	GTTTCTACTC	ACAAAATCAC	ATAAGAAAGC	GGAGAAAGCA	AAATCCAACC
1251	TGCCTTCGTA	GTGGCATTAC	GTATTTTACC	CGTTTAATGG	AAACTTCCTC
	ACGGAAGCAT	CACCGTAATG	CATAAAATGG	GCAAATTACC	TTTGAAGGAG
1301	ATGCGTAAGT	CTTTAGTCCT	CAAAGCCTCC	GTAGCCGTTG	CTACCCTCGT
	TACGCATTCA	GAAATCAGGA	GTTTCGGAGG	CATCGGCAAC	GATGGGAGCA
1351	TCCGATGCTG	TCTTTCGCTG	CTGAGGGTGA	CGATCCCGCA	AAAGCGGCCT
	AGGCTACGAC	AGAAAGCGAC	GACTCCCACT	GCTAGGGCGT	TTTCGCCGGA
1401	TTGACTCCCT	GCAAGCCTCA	GCGACCGAAT	ATATCGGTTA	TGCGTGGGCG
	AACTGAGGGA	CGTTCGGAGT	CGCTGGCTTA	TATAGCCAAT	ACGCACCCGC
1451	ATGGTTGTTG	TCATTGTCGG	CGCAACTATC	GGTATCAAGC	TGTTTAAGAA
	TACCAACAAC	AGTAACAGCC	GCGTTGATAG	CCATAGTTCTG	ACAAATTCTT
1501	ATTCACCTCG	AAAGCAAGCT	GATAAAGGAG	GTTTCTCGAT	CGAGACGTTN
	TAAGTGGAGC	TTTCGTTCTGA	CTATTTCTCTC	CAAAGAGCTA	GCTCTGCAAN
1551	NNNGAGGTTC	CAACTTTCAC	CATAATGAAA	TAAGATCACT	ACCGGGCGTA
	NNNCTCCAAG	GTTGAAAGTG	GTATTACTTT	ATTCTAGTGA	TGGCCCGCAT
1601	TTTTTTGAGT	TATCGAGATT	TTCAGGAGCT	AAGGAAGCTA	AAATGGAGAA
	AAAAAATCA	ATAGCTCTAA	AAGTCCTCGA	TTCTTTCGAT	TTTACCTCTT
1651	AAAAATCACT	GGATATACCA	CCGTTGATAT	ATCCCAATGG	CATCGTAAAG
	TTTTTAGTGA	CCTATATGGT	GGCAACTATA	TAGGGTTACC	GTAGCATTTT

6/39

1701	AACATTTTGA	GGCATTTTCAG	TCAGTTGCTC	AATGTACCTA	TAACCAGACC
	TTGTAAACT	CCGTAAAGTC	AGTCAACGAG	TTACATGGAT	ATTGGTCTGG
1751	G TTCAGCTGG	ATATTACGGC	CTTTTTTAAAG	ACCGTAAAGA	AAAATAAGCA
	CAAGTCGACC	TATAATGCCG	GAAAAATTTC	TGGCATTCT	TTTTATTCTG
1801	CAAGTTTTAT	CCGGCCTTTA	TTCACATTCT	TGCCCCGCTG	ATGAATGCTC
	GTTCAAAATA	GGCCGGAAAT	AAGTGTAAGA	ACGGGCGGAC	TACTTACGAG
1851	ATCCGGAGTT	CCGTATGGCA	ATGAAAGACG	GTGAGCTGGT	GATATGGGAT
	TAGGCCTCAA	GGCATACCGT	TACTTTCTGC	CACTCGACCA	CTATACCCTA
1901	AGTGTTTACC	CTTGTTACAC	CGTTTTCCAT	GAGCAAACCTG	AAACGTTTTTC
	TCACAAGTGG	GAACAATGTG	GCAAAAGGTA	CTCGTTTGAC	TTTGCAAAAG
1951	ATCGCTCTGG	AGTGAATACC	ACGACGATTT	CCGGCAGTTT	CTACACATAT
	TAGCGAGACC	TCACTTATGG	TGCTGCTAAA	GGCCGTCAAA	GATGTGTATA
2001	ATTCGCAAGA	TGTGGCGTGT	TACGGTGAAA	ACCTGGCCTA	TTTCCCTAAA
	TAAGCGTTCT	ACACCGCACA	ATGCCACTTT	TGGACCGGAT	AAAGGGATTT
2051	GGGTTTATTG	AGAATATGTT	TTTCGTCTCA	GCCAATCCCT	GGGTGAGTTT
	CCCAAATAAC	TCTTATACAA	AAAGCAGAGT	CGGTTAGGGA	CCCACTCAAA
2101	CACCAGTTTT	GATTTAAACG	TGGCCAATAT	GGACAACTTC	TTCGCCCCCG
	GTGGTCAAAA	CTAAATTTGC	ACCGGTTATA	CCTGTTGAAG	AAGCGGGGGC

NcoI

~~~~~

|      |             |             |            |            |             |
|------|-------------|-------------|------------|------------|-------------|
| 2151 | TTTTCAACCAT | GGGCAAATAT  | TATACGCAAG | GCGACAAGGT | GCTGATGCCG  |
|      | AAAAGTGGTA  | CCCGTTTATA  | ATATGCGTTC | CGCTGTTCCA | CGACTACGGC  |
| 2201 | CTGGCGATTTC | AGGTTCATCA  | TGCCGTCTGT | GATGGCTTCC | ATGTCGGCAG  |
|      | GACCGCTAAG  | TCCAAGTAGT  | ACGGCAGACA | CTACCGAAGG | TACAGCCGTC  |
| 2251 | AATGCTTAAT  | GAATTACAAC  | AGTACTGCGA | TGAGTGGCAG | GGCGGGGCGT  |
|      | TTACGAATTA  | CTTAATGTTG  | TCATGACGCT | ACTCACCGTC | CCGCCCCGCA  |
| 2301 | AATTTTTTTTA | AGGCAGTTAT  | TGGTGCCCTT | AAACGCCTGG | TGCTACGCCT  |
|      | TTAAAAAAAT  | TCCGTCAATA  | ACCACGGGAA | TTTGCGGACC | ACGATGCGGA  |
| 2351 | GAATAAGTGA  | TAATAAGCGG  | ATGAATGGCA | GAAATTCGAA | AGCAAATTCTG |
|      | CTTATTCACT  | ATTATTTCGCC | TACTTACCGT | CTTTAAGCTT | TCGTTTAAGC  |
| 2401 | ACCCGGTTCGT | CGGTTTCAGGG | CAGGGTCGTT | AAATAGCCGC | TTATGTCTAT  |
|      | TGGGCCAGCA  | GCCAAGTCCC  | GTCCCAGCAA | TTTATCGGCG | AATACAGATA  |
| 2451 | TGCTGGTTTA  | CCGGTTTATT  | GACTACCGGA | AGCAGTGTGA | CCGTGTGCTT  |
|      | ACGACCAAAT  | GGCCAAATAA  | CTGATGGCCT | TCGTCACACT | GGCACACGAA  |
| 2501 | CTCAAATGCC  | TGAGGCCAGT  | TTGCTCAGGC | TCTCCCCGTG | GAGGTAATAA  |
|      | GAGTTTACGG  | ACTCCGGTCA  | AACGAGTCCG | AGAGGGGCAC | CTCCATTATT  |

7/39

|      |             |             |             |            |            |
|------|-------------|-------------|-------------|------------|------------|
| 2551 | TTGCTCGACC  | GATAAAAGCG  | GCTTCCTGAC  | AGGAGGCCGT | TTTGTTTTGC |
|      | AACGAGCTGG  | CTATTTTCGC  | CGAAGGACTG  | TCCTCCGGCA | AAACAAAACG |
| 2601 | AGCCACCTC   | AACGCAATTA  | ATGTGAGTTA  | GCTCACTCAT | TAGGCACCCC |
|      | TCGGGTGGAG  | TTGCGTTAAT  | TACACTCAAT  | CGAGTGAGTA | ATCCGTGGGG |
| 2651 | AGGCTTTTACA | CTTTATGCTT  | CCGGCTCGTA  | TGTTGTGTGG | AATTGTGAGC |
|      | TCCGAAATGT  | GAAATACGAA  | GGCCGAGCAT  | ACAACACACC | TTAACACTCG |
| 2701 | GGATAACAAT  | TTCACACAGG  | AAACAGCTAT  | GACCATGATT | ACGAATTTCT |
|      | CCTATTGTTA  | AAGTGTGTCC  | TTTGTGCGATA | CTGGTACTAA | TGCTTAAAGA |
| 2751 | AGATAACGAG  | GGCAAATCAT  | GAAAAAGACA  | GCTATCGCGA | TTGCAGTGGC |
|      | TCTATTGCTC  | CCGTTTAGTA  | CTTTTTCTGT  | CGATAGCGCT | AACGTCACCG |
| 2801 | ACTGGCTGGT  | TTGCTACCG   | TAGCGCAGGC  | CGACTACAAA | GATATCGTTA |
|      | TGACCGACCA  | AAGCGATGGC  | ATCGCGTCCG  | GCTGATGTTT | CTATAGCAAT |
| 2851 | TGACCCAGTC  | ACCGTCCTCC  | CTGACCGTTA  | CCGCTGGTGA | AAAAGTTACC |
|      | ACTGGGTCAG  | TGGCAGGAGG  | GACTGGCAAT  | GGCGACCACT | TTTTCAATGG |
| 2901 | ATGTCCTGCA  | CCTCCTCCCA  | GTCCCTGTTC  | AACTCCGGTA | AACAGAAAAA |
|      | TACAGGACGT  | GGAGGAGGGT  | CAGGGACAAG  | TTGAGGCCAT | TTGTCTTTTT |
| 2951 | CTACCTGACC  | TGGTATCAGC  | AGAAACCGGG  | TCAGCCACCG | AAAGTTCTGA |
|      | GATGGACTGG  | ACCATAGTCG  | TCTTTGGCCC  | AGTCGGTGGC | TTTCAAGACT |
| 3001 | TCTACTGGGC  | TTCCACCCGT  | GAATCCGGTG  | TTCCAGACCG | TTTCACCGGT |
|      | AGATGACCCG  | AAGGTGGGCA  | CTTAGGCCAC  | AAGGTCTGGC | AAAGTGGCCA |
| 3051 | TCCGGTTCCG  | GCACCGACTT  | CACCCTGACC  | ATCTCCTCCG | TTCAGGCTGA |
|      | AGGCCAAGGC  | CGTGGCTGAA  | GTGGGACTGG  | TAGAGGAGGC | AAGTCCGACT |
| 3101 | AGACCTGGCT  | GTTTACTACT  | GCCAGAACGA  | CTACTCCAAC | CCACTGACCT |
|      | TCTGGACCGA  | CAAATGATGA  | CGGTCTTGCT  | GATGAGGTTG | GGTGACTGGA |
| 3151 | TCGGTGGTGG  | CACCAAACCTG | GAACCTTAAGC | GCGCTGGTGG | TGGAGGGTCT |
|      | AGCCACCACC  | GTGGTTTGAC  | CTTGAATTCG  | CGCGACCACC | ACCTCCCAGA |
|      |             |             | BamHI       |            |            |
|      |             |             | ~~~~~       |            |            |
| 3201 | GGAGGAGGTG  | GGAGTGGGGG  | AGGTGGATCC  | GGCGGGGGAG | GTTCAGGGGG |
|      | CCTCCTCCAC  | CCTCACCCCC  | TCCACCTAGG  | CCGCCCCCTC | CAAGTCCCCC |
| 3251 | TGGCGGTAGT  | GGAGGGGGCG  | GTTCAGAAGT  | TCAACTAGTT | GAATCCGGTG |
|      | ACCGCCATCA  | CCTCCCCCGC  | CAAGTCTTCA  | AGTTGATCAA | CTTAGGCCAC |
| 3301 | GTGACCTGGT  | TAAACCGGGT  | GGTTCCTTGA  | AACTGTCCTG | CGCTGCTTCC |
|      | CACTGGACCA  | ATTTGGCCCC  | CCAAGGGACT  | TTGACAGGAC | GCGACGAAGG |

8/39

3351 GGTTCCTCCT TCTCCTCCTA CGGTATGTCC TGGGTTCGTC AGACCCCGGA  
 CCAAAGAGGA AGAGGAGGAT GCCATACAGG ACCCAAGCAG TCTGGGGCCT

3401 CAAACGTCTG GAATGGGTTG CTACCATCTC CAACGGTGGT GGTTACACCT  
 GTTTGCAGAC CTTACCCAAC GATGGTAGAG GTTGCCACCA CCAATGTGGA

3451 ACTACCCGGA CTCCGTTAAA GGTCGTTTCA CCATCTCCCG TGACAACGCT  
 TGATGGGCCT GAGGCAATTT CCAGCAAAGT GGTAGAGGGC ACTGTTGCGA

PstI

~~~~~

3501 AAAAACACCC TGTACCTGCA GATGTCCTCC CTGAAATCCG AAGACTCAGC  
 TTTTGTGGG ACATGGACGT CTACAGGAGG GACTTTAGGC TTCTGAGTCG

3551 TATGTACTAC TGCGCTCGTC GTGAACGTTA CGACGAAAAC GGTTTCGCTT  
 ATACATGATG ACGCGAGCAG CACTTGCAAT GCTGCTTTTG CCAAAGCGAA

EcoRI

~~~~~

3601 ACTGGGGTCA GGGTACCCTG GTTACCGTTT CAGCTTCCGG AGAATTCGAG  
 TGACCCAGT CCCATGGGAC CAATGGCAAA GTCGAAGGCC TCTTAAGCTC

AvaI

~~~~~

3651 GCCTCGGGGG CCGAGGGCGG CGGTTCTGGT TCCGGTGATT TTGATTATGA  
 CGGAGCCCCC GGCTCCCGCC GCCAAGACCA AGGCCACTAA AACTAATACT

3701 AAAAATGGCA AACGCTAATA AGGGGGCTAT GACCGAAAAT GCCGATGAAA  
 TTTTACCCTG TTGCGATTAT TCCCCGATA CTGGCTTTTA CGGCTACTTT

3751 ACGCGCTACA GTCTGACGCT AAAGGCAAAC TTGATTCTGT CGCTACTGAT  
 TGCGCGATGT CAGACTGCGA TTTCCGTTTG AACTAAGACA GCGATGACTA

ClaI

~~~~~

3801 TACGGTGCTG CTATCGATGG TTTCATTGGT GACGTTTCCG GCCTTGCTAA  
 ATGCCACGAC GATAGCTACC AAAGTAACCA CTGCAAAGGC CGGAACGATT

3851 TGGTAATGGT GCTACTGGTG ATTTTGCTGG CTCTAATTCC CAAATGGCTC  
 ACCATTACCA CGATGACCAC TAAAACGACC GAGATTAAGG GTTTACCGAG

3901 AAGTCGGTGA CGGTGATAAT TCACCTTTAA TGAATAATTT CCGTCAATAT  
 TTCAGCCACT GCCACTATTA AGTGGAATTT ACTTATTAAA GGCAGTTATA

3951 TTACCTTCCC TCCCTCAATC GGTTGAATGT CGCCCTTTTG TCTTTGGCGC  
 AATGGAAGGG AGGGAGTTAG CCAACTTACA GCGGGAAAAC AGAAACCGCG

4001 TGGTAAACCA TATGAATTTT CTATTGATTG TGACAAAATA AACTTATTCC  
 ACCATTGGT ATACTTAAAA GATAACTAAC ACTGTTTTAT TTGAATAAGG

4051 GTGGTGTCTT TGCGTTTCTT TTATATGTTG CCACCTTTAT GTATGTATTT  
 CACCACAGAA ACGCAAAGAA AATATACAAC GGTGGAAATA CATAcataaa

9/39

HindIII

~~~~~

4101	TCTACGTTTG	CTAACATACT	GCGTAATAAG	GAGTCTTGAT	AAGCTTCGAG
	AGATGCAAAC	GATTGTATGA	CGCATTATTC	CTCAGAACTA	TTCGAAGCTC
4151	AAATTCACCT	CGAAAGCAAG	CTGATAAACC	GATACAATTA	AAGGCTCCTT
	TTTAAGTGGA	GCTTTCGTTC	GACTATTTGG	CTATGTTAAT	TTCCGAGGAA

EcoRI

~~~~~

|      |            |             |             |            |             |
|------|------------|-------------|-------------|------------|-------------|
| 4201 | TTGGAGCCTT | TTTTTTTGGA  | GAATTC AATC | ATGCCAGTTC | TTTTGGGTAT  |
|      | AACCTCGGAA | AAAAAAACCT  | CTTAAGTTAG  | TACGGTCAAG | AAAACCCATA  |
| 4251 | TCCGTTATTA | TTGCGTTTCC  | TCGGTTTCCT  | TCTGGTAACT | TTGTTCCGGCT |
|      | AGGCAATAAT | AACGCAAAGG  | AGCCAAAGGA  | AGACCATTGA | AACAAGCCGA  |
| 4301 | ATCTGCTTAC | TTTCCTTAAA  | AAGGGCTTCG  | GTAAGATAGC | TATTGCTATT  |
|      | TAGACGAATG | AAAGGAATTT  | TTCCCGAAGC  | CATTCTATCG | ATAACGATAA  |
| 4351 | TCATTGTTTC | TTGCTCTTAT  | TATTGGGCTT  | AACTCAATTC | TTGTGGGTTA  |
|      | AGTAACAAAG | AACGAGAATA  | ATAACCCGAA  | TTGAGTTAAG | AACACCCAAT  |
| 4401 | TCTCTCTGAT | ATTAGCGCAC  | AATTACCCTC  | TGATTTTGTT | CAGGGCGTTC  |
|      | AGAGAGACTA | TAATCGCGTG  | TTAATGGGAG  | ACTAAAACAA | GTCCCGCAAG  |
| 4451 | AGTTAATTCT | CCCGTCTAAT  | GCGCTTCCCT  | GTTTTTATGT | TATTCTCTCT  |
|      | TCAATTAAGA | GGGCAGATTA  | CGCGAAGGGA  | CAAAAATACA | ATAAGAGAGA  |
| 4501 | GTAAAGGCTG | CTATTTTTCAT | TTTTGACGTT  | AAACAAAAAA | TCGTTTCTTA  |
|      | CATTTCCGAC | GATAAAAGTA  | AAAACCTGCA  | TTTGTTTTTT | AGCAAAGAAT  |
| 4551 | TTTGGATTGG | GATAAATAAA  | TATGGCTGTT  | TATTTTGTA  | CTGGCAAATT  |
|      | AAACCTAACC | CTATTTATTT  | ATACCGACAA  | ATAAAACATT | GACCGTTTAA  |
| 4601 | AGGCTCTGGA | AAGACGCTCG  | TTAGCGTTGG  | TAAGATTCAG | GATAAAATTG  |
|      | TCCGAGACCT | TTCTGCGAGC  | AATCGCAACC  | ATTCTAAGTC | CTATTTTAAC  |
| 4651 | TAGCTGGGTG | CAAAATAGCA  | ACTAATCTTG  | ATTTAAGGCT | TCAAAACCTC  |
|      | ATCGACCCAC | GTTTTATCGT  | TGATTAGAAC  | TAAATTCCGA | AGTTTTGGAG  |
| 4701 | CCGCAAGTCG | GGAGGTTTCG  | TAAAACGCCT  | CGCGTTCTTA | GAATACCGGA  |
|      | GGCGTTCAGC | CCTCCAAGCG  | ATTTTGCGGA  | GCGCAAGAAT | CTTATGGCCT  |
| 4751 | TAAGCCTTCT | ATTTCTGATT  | TGCTTGCTAT  | TGGTCGTGGT | AATGATTTCCT |
|      | ATTCGGAAGA | TAAAGACTAA  | ACGAACGATA  | ACCAGCACCA | TTACTAAGGA  |
| 4801 | ACGACGAAAA | TAAAAACGGT  | TTGCTTGTTT  | TTGATGAATG | CGGTACTTGG  |
|      | TGCTGCTTTT | ATTTTTGCCA  | AACGAACAAG  | AACTACTTAC | GCCATGAACC  |
| 4851 | TTTAATACCC | GTTTATGGAA  | TGACAAGGAA  | AGACAGCCGA | TTATTGATTG  |
|      | AAATTATGGG | CAAGTACCTT  | ACTGTTTCCT  | TCTGTCGGCT | AATAACTAAC  |

10/39

|      |                          |                           |                          |                          |                           |
|------|--------------------------|---------------------------|--------------------------|--------------------------|---------------------------|
| 4901 | GTTTCTTCAT<br>CAAAGAAGTA | GCTCGTAAAT<br>CGAGCATTTA  | TGGGATGGGA<br>ACCCTACCT  | TATTATTTTT<br>ATAATAAAAA | CTTGTTTCAGG<br>GAACAAGTCC |
| 4951 | ATTTATCTAT<br>TAAATAGATA | TGTTGATAAA<br>ACAAC TATTT | CAGGCGCGTT<br>GTCCGCGCAA | CTGCATTAGC<br>GACGTAATCG | TGAACACGTT<br>ACTTGTGCAA  |
| 5001 | GTTTATTGTC<br>CAAATAACAG | GCCGTCTGGA<br>CGGCAGACCT  | CAGAATTACT<br>GTCTTAATGA | TTACCCTTTG<br>AATGGGAAAC | TCGGCACTTT<br>AGCCGTGAAA  |
| 5051 | ATATTCTCTT<br>TATAAGAGAA | GTTACTGGCT<br>CAATGACCGA  | CAAAAATGCC<br>GTTTTTACGG | TCTGCCTAAA<br>AGACGGATTT | TTACATGTTG<br>AATGTACAAC  |
| 5101 | GTGTTGTTAA<br>CACAACAATT | ATATGGTGAT<br>TATACCACTA  | TCTCAATTAA<br>AGAGTTAATT | GCCCTACTGT<br>CGGGATGACA | TGAGCGTTGG<br>ACTCGCAACC  |
| 5151 | CTTTATACTG<br>GAAATATGAC | GTAAGAATTT<br>CATTCTTAAA  | ATATAACGCA<br>TATATTGCGT | TATGACACTA<br>ATACTGTGAT | AACAGGCTTT<br>TTGTCCGAAA  |
| 5201 | TTCCAGTAAT<br>AAGGTCATTA | TATGATTCAG<br>ATACTAAGTC  | GTGTTTATTC<br>CACAAATAAG | ATATTTAACC<br>TATAAATTGG | CCTTATTTAT<br>GGAATAAATA  |
| 5251 | CACACGGTCG<br>GTGTGCCAGC | GTATTTCAAA<br>CATAAAGTTT  | CCATTAAATT<br>GGTAATTTAA | TAGGTCAGAA<br>ATCCAGTCTT | GATGAAATTA<br>CTACTTTAAT  |
| 5301 | ACTAAAATAT<br>TGATTTTATA | ATTTGAAAAA<br>TAAACTTTTT  | GTTTTCTCGC<br>CAAAAGAGCG | GTTCTTTGTC<br>CAAGAAACAG | TTGCGATAGG<br>AACGCTATCC  |
| 5351 | ATTTGCATCA<br>TAAACGTAGT | GCATTTACAT<br>CGTAAATGTA  | ATAGTTATAT<br>TATCAATATA | AACCCAACCT<br>TTGGGTTGGA | AAGCCGGAGG<br>TTCGGCCTCC  |
| 5401 | TTAAAAAGGT<br>AATTTTTCCA | AGTCTCTCAG<br>TCAGAGAGTC  | ACCTATGATT<br>TGGATACTAA | TTGATAAATT<br>AACTATTTAA | CACTATTGAC<br>GTGATAACTG  |
| 5451 | TCTTCTCAGC<br>AGAAGAGTCG | GTCTTAATCT<br>CAGAATTAGA  | AAGCTATCGC<br>TTCGATAGCG | TATGTTTTCA<br>ATACAAAAGT | AGGATTCTAA<br>TCCTAAGATT  |
| 5501 | GGGAAAATTA<br>CCCTTTTAAT | ATTAATAGCG<br>TAATTATCGC  | ACGATTTACA<br>TGCTAAATGT | GAAGCAAGGT<br>CTTCGTTCCA | TATTCCATCA<br>ATAAGGTAGT  |
| 5551 | CATATATTGA<br>GTATATAACT | TTTATGTACT<br>AAATACATGA  | GTTTCAATTA<br>CAAAGTTAAT | AAAAAGGTAA<br>TTTTTCCATT | TTCAAATGAA<br>AAGTTTACTT  |
| 5601 | ATTGTTAAAT<br>TAACAATTTA | GTAATTAATT<br>CATTAATTAA  | TTGTTTTCTT<br>AACAAAAGAA | GATGTTTGTT<br>CTACAAACAA | TCATCATCTT<br>AGTAGTAGAA  |
| 5651 | CTTTTGCTCA<br>GAAAACGAGT | AGTAATTGAA<br>TCATTAACTT  | ATGAATAATT<br>TACTTATTAA | CGCCTCTGCG<br>GCGGAGACGC | CGATTTTCGTG<br>GCTAAAGCAC |
| 5701 | ACTTGGTATT<br>TGAACCATAA | CAAAGCAAAC<br>GTTTCGTTTG  | AGGTGAATCT<br>TCCACTTAGA | GTTATTGTCT<br>CAATAACAGA | CACCTGATGT<br>GTGGACTACA  |

11/39

|      |            |            |            |            |            |
|------|------------|------------|------------|------------|------------|
| 5751 | TAAAGGTACA | GTGACTGTAT | ATTCCTCTGA | CGTTAAGCCT | GAAAATTTAC |
|      | ATTTCCATGT | CACTGACATA | TAAGGAGACT | GCAATTCGGA | CTTTTAAATG |
| 5801 | GCAATTTCTT | TATCTCTGTT | TTACGTGCTA | ATAATTTTGA | TATGGTTGGC |
|      | CGTTAAAGAA | ATAGAGACAA | AATGCACGAT | TATTAAAACT | ATACCAACCG |
| 5851 | TCAATTCCTT | CCATAATTCA | GAAATATAAC | CCAAATAGTC | AGGATTATAT |
|      | AGTTAAGGAA | GGTATTAAGT | CTTTATATTG | GGTTTATCAG | TCCTAATATA |
| 5901 | TGATGAATTG | CCATCATCTG | ATATTCAGGA | ATATGATGAT | AATTCCGCTC |
|      | ACTACTTAAC | GGTAGTAGAC | TATAAGTCCT | TATACTACTA | TTAAGGCGAG |
| 5951 | CTTCTGGTGG | TTTCTTTGTT | CCGCAAAATG | ATAATGTTAC | TCAAACATTT |
|      | GAAGACCACC | AAAGAAACAA | GGCGTTTTAC | TATTACAATG | AGTTTGTAAG |
| 6001 | AAAATTAATA | ACGTTTCGCG | AAAGGATTTA | ATAAGGGTTG | TAGAATTGTT |
|      | TTTTAATTAT | TGCAAGCGCG | TTTCCTAAAT | TATTCCTAAC | ATCTTAACAA |
| 6051 | TGTTAAATCT | AATACATCTA | AATCCTCAAA | TGTATTATCT | GTTGATGGTT |
|      | ACAATTTAGA | TTATGTAGAT | TTAGGAGTTT | ACATAATAGA | CACTACCAA  |
| 6101 | CTAACTTATT | AGTAGTTAGC | GCCCCTAAAG | ATATTTTAGA | TAACCTTCCG |
|      | GATTGAATAA | TCATCAATCG | CGGGGATTTT | TATAAAATCT | ATTGGAAGGC |
| 6151 | CAATTTCTTT | CTACTGTTGA | TTTGCCAACT | GACCAGATAT | TGATTGAAGG |
|      | GTTAAAGAAA | GATGACAAC  | AAACGGTTGA | CTGGTCTATA | ACTAACTTCC |
| 6201 | ATTAATTTTC | GAGGTTTCAG | AAGGTGATGC | TTTAGATTTT | TCCTTTGCTG |
|      | TAATTTAAAG | CTCCAAGTCG | TTCCACTACG | AAATCTAAAA | AGGAAACGAC |
| 6251 | CTGGCTCTCA | GCGCGGCACT | GTTGCTGGTG | GTGTTAATAC | TGACCGTCTA |
|      | GACCGAGAGT | CGCGCCGTGA | CAACGACCAC | CACAATTATG | ACTGGCAGAT |
| 6301 | ACCTCTGTTT | TATCTTCTGC | GGGTGGTTTC | TTCGGTATTT | TTAACGGCGA |
|      | TGGAGACAAA | ATAGAAGACG | CCCACCAAGC | AAGCCATAAA | AATTGCCGCT |
| 6351 | TGTTTTAGGG | CTATCAGTTC | GCGCATTAAA | GACTAATAGC | CATTCAAAAA |
|      | ACAAAATCCC | GATAGTCAAG | CGCGTAATTT | CTGATTATCG | GTAAGTTTTT |
| 6401 | TATTGTCTGT | GCCTCGTATT | CTTACGCTTT | CAGGTCAGAA | GGGTTCTATT |
|      | ATAACAGACA | CGGAGCATAA | GAATGCGAAA | GTCCAGTCTT | CCCAAGATAA |
| 6451 | TCTGTTGGCC | AGAATGTCCC | TTTTATTACT | GGTCGTGTAA | CTGGTGAATC |
|      | AGACAACCGG | TCTTACAGGG | AAAATAATGA | CCAGCACATT | GACCACTTAG |
| 6501 | TGCCAATGTA | AATAATCCAT | TTCAGACGGT | TGAGCGTCAA | AATGTTGGTA |
|      | ACGGTTACAT | TTATTAGGTA | AAGTCTGCCA | ACTCGCAGTT | TTACAACCAT |
| 6551 | TTTCTATGAG | TGTTTTTCCC | GTTGCAATGG | CTGGCGGTAA | TATTGTTTTA |
|      | AAAGATACTC | ACAAAAGGGG | CAACGTTACC | GACCGCCATT | ATAACAAAAT |



12/39

6601 GATATAACCA GTAAGGCCGA TAGTTTGAGT TCTTCTACTC AGGCAAGTGA  
CTATATTGGT CATTCCGGCT ATCAAACCTCA AGAAGATGAG TCCGTTCACT

6651 TGTTATTACT AATCAAAGAA GTATTGCGAC AACGGTTAAT TTGCGTGATG  
ACAATAATGA TTAGTTTCTT CATAACGCTG TTGCCAATTA AACGCACTAC

6701 GTCAGACTCT TTTGCTCGGT GGCCTCACTG ATTACAAAAA CACTTCTCAA  
CAGTCTGAGA AAACGAGCCA CCGGAGTGAC TAATGTTTTT GTGAAGAGTT

6751 GATTCTGGTG TGCCGTTTCT GTCTAAAATC CCTTTAATCG GCCTCCTGTT  
CTAAGACCAC ACGGCAAGGA CAGATTTTAG GGAAATTAGC CGGAGGACAA

6801 TAGCTCCCGT TCTGATTCTA ACGAGGAAAG CACGTTGTAC GTGCTCGTCA  
ATCGAGGGCA AGACTAAGAT TGCTCCTTTC GTGCAACATG CACGAGCAGT

6851 AAGCAACCAT AGTACGCGCC CTGTAGCGGC GCATTAAGCG CGGCGGGTGT  
TTCGTTGGTA TCATGCGCGG GACATCGCCG CGTAATTTCG GCCGCCACACA

6901 GGTGGTTACG CGCAGCGTGA CCGCTACACT TGCCAGCGCC CTAGCGCCCG  
CCACCAATGC GCGTCGCACT GGCGATGTGA ACGGTCGCGG GATCGCGGGC

6951 CTCCTTTCGC TTTCTTCCCT TCCTTTCTCG CCACGTTCTC CGGCTTTCCC  
GAGGAAAGCG AAAGAAGGGA AGGAAAGAGC GGTGCAAGAG GCCGAAAGGG

BamHI

~~~~~

7001 CGTCAAGCTC TAAATCGGGG GATCCCTTTA GGGTTCCGAT TTAGTGCTTT  
GCAGTTCGAG ATTTAGCCCC CTAGGGAAAT CCCAAGGCTA AATCACGAAA

7051 ACGGCACCTC GACCTCCAAA AACTTGATTT GGGTGATGGT TCACGTAGTG  
TGCCGTGGAG CTGGAGGTTT TTGAACTAAA CCCACTACCA AGTGCATCAC

7101 GGCCATCGCC CTGATAGACG GTTTTTTCGCC CTTTGACGTT GGAGTCCACG  
CCGGTAGCGG GACTATCTGC CAAAAAGCGG GAAACTGCAA CCTCAGGTGC

7151 TTCTTTAATA GTGGACTCTT GTTCCAACT GGAACAACAC TCACAATAA  
AAGAAATTAT CACCTGAGAA CAAGGTTTGA CCTTGTTGTG AGTGTTGATT

7201 CTCGGCCTAT TCTTTTGATT TATAAGGATT TTTGTCATTT TCTGCTTACT  
GAGCCGGATA AGAAAACTAA ATATTCCTAA AAACAGTAAA AGACGAATGA

7251 GGTTAACAAA TAAGCTGATT TAACAAATAT TTAACGCGAA ATTTAACAAA  
CCAATTTTTT ATTCGACTAA ATTGTTTATA AATTGCGCTT TAAATTGTTT

7301 ACATTAACGT TTACAATTTA AATATTTGCT TATACAATCA TCCTGTTTTT  
TGTAATTGCA AATGTTAAAT TTATAAACGA ATATGTTAGT AGGACAAAAA

7351 GGGGCTTTTC TGATTATCAA CCGGGGTACA TATGATTGAC ATGCTAGTTT  
CCCCGAAAAG ACTAATAGTT GGCCCCATGT ATACTAACTG TACGATCAAA

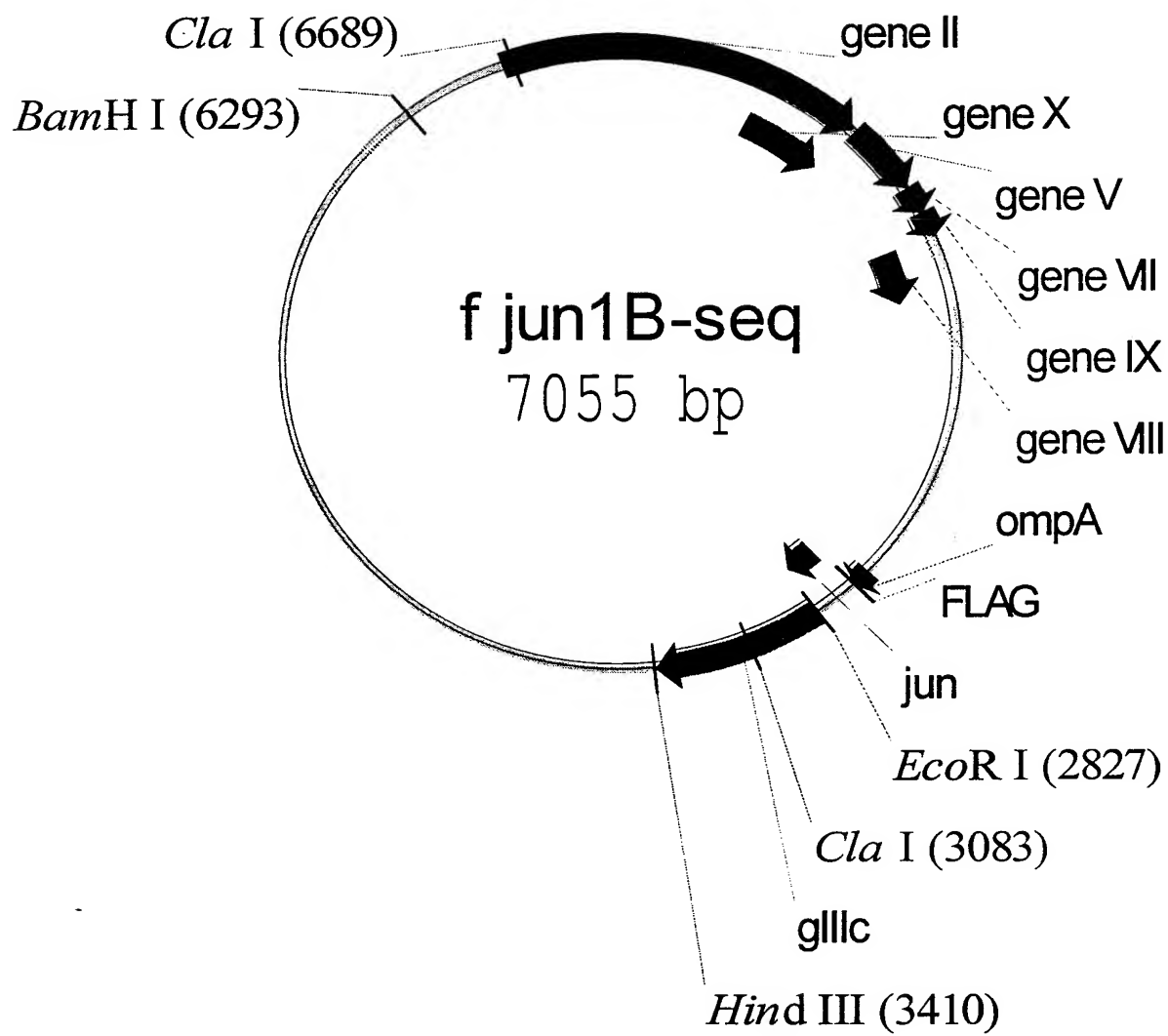
13/39

Clal

~~~~~

|      |            |             |            |            |             |
|------|------------|-------------|------------|------------|-------------|
| 7401 | TACGATTACC | G TTCATCGAT | TCTCTTGTTT | GCTCCAGACT | TTCAGGTAAT  |
|      | ATGCTAATGG | CAAGTAGCTA  | AGAGAACAAA | CGAGGTCTGA | AAGTCCATTA  |
| 7451 | GACCTGATAG | CCTTTGTAGA  | CCTCTCAAAA | ATAGCTACCC | TCTCCGGCAT  |
|      | CTGGACTATC | GGAAACATCT  | GGAGAGTTTT | TATCGATGGG | AGAGGCCGTA  |
| 7501 | GAATTTATCA | GCTAGAACGG  | TTGAATATCA | TATTGACGGT | GATTTGACTG  |
|      | CTTAAATAGT | CGATCTTGCC  | AACTTATAGT | ATAACTGCCA | CTAAACTGAC  |
| 7551 | TCTCCGGCCT | TTCTCACCCG  | TTTGAATCTT | TGCCTACTCA | T TACTCCGGC |
|      | AGAGGCCGGA | AAGAGTGGGC  | AAACTTAGAA | ACGGATGAGT | AATGAGGCCG  |
| 7601 | ATTGCATTTA | AAATATATGA  | GGGTTCTAAA | AATTTTTATC | CCTGCGTTGA  |
|      | TAACGTAAAT | TTTATATACT  | CCCAAGATTT | TTAAAAATAG | GGACGCAACT  |
| 7651 | AATTAAGGCT | TCACCAGCAA  | AAGTATTACA | GGGTCATAAT | GTTTTTGGTA  |
|      | TTAATTCCGA | AGTGGTCGTT  | TTCATAATGT | CCCAGTATTA | CAAAAACCAT  |
| 7701 | CAACCGATTT | AGCTTTATGC  | TCTGAGGCTT | TATTGCTTAA | TTTTGCTAAC  |
|      | GTTGGCTAAA | TCGAAATACG  | AGACTCCGAA | ATAACGAATT | AAAACGATTG  |
| 7751 | TCTCTGCCTT | GCTTGTACGA  | TTTATTGGAT | GTT        |             |
|      | AGAGACGGAA | CGAACATGCT  | AAATAACCTA | CAA        |             |

14/39

**Figure 3**

15/39

|     |            |            |            |             |            |
|-----|------------|------------|------------|-------------|------------|
| 1   | AACGCTACTA | CCATTAGTAG | AATTGATGCC | ACCTTTTTCAG | CTCGCGCCCC |
|     | TTGCGATGAT | GGTAATCATC | TTAACTACGG | TGGAAAAGTC  | GAGCGCGGGG |
| 51  | AAATGAAAAT | ATAGCTAAAC | AGGTTATTGA | CCATTTGCGA  | AATGTATCTA |
|     | TTTACTTTTA | TATCGATTTG | TCCAATAACT | GGTAAACGCT  | TTACATAGAT |
| 101 | ATGGTCAAAC | TAAATCTACT | CGTTCGCAGA | ATTGGGAATC  | AACTGTTACA |
|     | TACCAGTTTG | ATTTAGATGA | GCAAGCGTCT | TAACCCTTAG  | TTGACAATGT |
| 151 | TGGAATGAAA | CTTCCAGACA | CCGTACTTTA | GTTGCATATT  | TAAAACATGT |
|     | ACCTTACTTT | GAAGGTCTGT | GGCATGAAAT | CAACGTATAA  | ATTTTGTACA |
| 201 | TGAACTACAG | CACCAGATTC | AGCAATTAAG | CTCTAAGCCA  | TCCGCAAAAA |
|     | ACTTGATGTC | GTGGTCTAAG | TCGTTAATTC | GAGATTCCGT  | AGGCGTTTTT |
| 251 | TGACCTCTTA | TCAAAAGGAG | CAATTAAAGG | TACTGTCTAA  | TCCTGACCTG |
|     | ACTGGAGAAT | AGTTTTCTCT | GTTAATTTCC | ATGACAGATT  | AGGACTGGAC |
| 301 | TTGGAATTTG | CTTCCGGTCT | GGTTCGCTTT | GAGGCTCGAA  | TTGAAACGCG |
|     | AACCTTAAAC | GAAGGCCAGA | CCAAGCGAAA | CTCCGAGCTT  | AACTTTGCGC |
| 351 | ATATTTGAAG | TCTTTCGGGC | TTCCTCTTAA | TCTTTTTGAT  | GCAATTCGCT |
|     | TATAAACTTC | AGAAAGCCCG | AAGGAGAATT | AGAAAACTA   | CGTTAAGCGA |
| 401 | TTGCTTCTGA | CTATAATAGA | CAGGGTAAAG | ACCTGATTTT  | TGATTTATGG |
|     | AACGAAGACT | GATATTATCT | GTCCCATTTT | TGGACTAAAA  | ACTAAATACC |
| 451 | TCATTCTCGT | TTTCTGAACT | GTTTAAAGCA | TTTGAGGGGG  | ATTCAATGAA |
|     | AGTAAGAGCA | AAAGACTTGA | CAAATTTCTG | AAACTCCCCC  | TAAGTTACTT |
| 501 | TATTTATGAC | GATTCCGCAG | TATTGGACGC | TATCCAGTCT  | AAACATTTTA |
|     | ATAAATACTG | CTAAGGCGTC | ATAACCTGCG | ATAGGTCAGA  | TTTGTAAAAT |
| 551 | CAATTACCCC | CTCTGGCAAA | ACTTCCTTTG | CAAAAGCCTC  | TCGCTATTTT |
|     | GTTAATGGGG | GAGACCGTTT | TGAAGGAAAC | GTTTTTCGGG  | AGCGATAAAA |
| 601 | GGTTTCTATC | GTCGTCTGGT | TAATGAGGGT | TATGATAGTG  | TTGCTCTTAC |
|     | CCAAAGATAG | CAGCAGACCA | ATTACTCCCA | ATACTATCAC  | AACGAGAATG |
| 651 | CATGCCTCGT | AATTCCTTTT | GGCGTTATGT | ATCTGCATTA  | GTTGAGTGTG |
|     | GTACGGAGCA | TTAAGGAAAA | CCGCAATACA | TAGACGTAAT  | CAACTCACAC |
| 701 | GTATTCCTAA | ATCTCAATTG | ATGAATCTTT | CCACCTGTAA  | TAATGTTGTT |
|     | CATAAGGATT | TAGAGTTAAC | TACTTAGAAA | GGTGGACATT  | ATTACAACAA |
| 751 | CCGTTAGTTC | GTTTTATTAA | CGTAGATTTT | TCCTCCCAAC  | GTCCTGACTG |
|     | GGCAATCAAG | CAAAATAATT | GCATCTAAAA | AGGAGGGTTG  | CAGGACTGAC |
| 801 | GTATAATGAG | CCAGTTCTTA | AAATCGCATA | AGGTAATTCA  | AAATGATTAA |
|     | CATATTACTC | GGTCAAGAAT | TTTAGCGTAT | TCCATTAAGT  | TTTACTAATT |

16/39

|      |             |              |               |             |             |
|------|-------------|--------------|---------------|-------------|-------------|
| 851  | AGTTGAAATT  | AAACCGTCTC   | AAGCGCAATT    | TACTACCCGT  | TCTGGTGTTT  |
|      | TCAACTTTAA  | TTTGGCAGAG   | TTCGCGTTAA    | ATGATGGGCA  | AGACCACAAA  |
| 901  | CTCGTCAGGG  | CAAGCCTTAT   | TCACTGAATG    | AGCAGCTTTG  | TTACGTTGAT  |
|      | GAGCAGTCCC  | GTTCGGAATA   | AGTGACTIONTAC | TCGTGGAAC   | AATGCAACTA  |
| 951  | TTGGGTAATG  | AATATCCGGT   | GCTTGTCAAG    | ATTACTCTCG  | ACGAAGGTCA  |
|      | AACCCATTAC  | TTATAGGCCA   | CGAACAGTTC    | TAATGAGAGC  | TGCTTCCAGT  |
| 1001 | GCCAGCGTAT  | GCGCCTGGTC   | TGTACACCGT    | GCATCTGTCC  | TCGTTCAAAG  |
|      | CGGTCGCATA  | CGCGGACCAG   | ACATGTGGCA    | CGTAGACAGG  | AGCAAGTTTC  |
| 1051 | TTGGTCAGTT  | CGGTTCTCTT   | ATGATTGACC    | GTCTGCGCCT  | CGTTCCGGCT  |
|      | AACCAGTCAA  | GCCAAGAGAA   | TACTAACTGG    | CAGACGCGGA  | GCAAGGCCGA  |
| 1101 | AAGTAACATG  | GAGCAGGTCG   | CGGATTTCTGA   | CACAATTTAT  | CAGGCGATGA  |
|      | TTCATTGTAC  | CTCGTCCAGC   | GCCTAAAGCT    | GTGTTAAATA  | GTCCGCTACT  |
| 1151 | TACAAATCTC  | CGTTGTACTT   | TGTTTCGCGC    | TTGGTATAAT  | CGCTGGGGGT  |
|      | ATGTTTAGAG  | GCAACATGAA   | ACAAAGCGCG    | AACCATATTA  | GCGACCCCCA  |
| 1201 | CAAAGATGAG  | TGTTTTAGTG   | TATTCTTTTCG   | CCTCTTTTCGT | TTTAGGTTGG  |
|      | GTTTCTACTC  | ACAAAATCAC   | ATAAGAAAGC    | GGAGAAAGCA  | AAATCCAACC  |
| 1251 | TGCCTTCGTA  | GTGGCATTAC   | GTATTTTACC    | CGTTTAATGG  | AAACTTCCTC  |
|      | ACGGAAGCAT  | CACCGTAATG   | CATAAAATGG    | GCAAATTACC  | TTTGAAGGAG  |
| 1301 | ATGCGTAAGT  | CTTTAGTCCT   | CAAAGCCTCC    | GTAGCCGTTG  | CTACCCCTCGT |
|      | TACGCATTCA  | GAAATCAGGA   | GTTTCGGAGG    | CATCGGCAAC  | GATGGGAGCA  |
| 1351 | TCCGATGCTG  | TCTTTCGCTG   | CTGAGGGTGA    | CGATCCCGCA  | AAAGCGGCCT  |
|      | AGGCTACGAC  | AGAAAGCGAC   | GACTIONCCACT  | GCTAGGGCGT  | TTTCGCCGGA  |
| 1401 | TTGACTCCCT  | GCAAGCCTCA   | GCGACCGAAT    | ATATCGGTTA  | TGCGTGGGCG  |
|      | AACTGAGGGA  | CGTTCGGAGT   | CGCTGGCTTA    | TATAGCCAAT  | ACGCACCCGC  |
| 1451 | ATGGTTGTTG  | TCATTGTCGG   | CGCAACTATC    | GGTATCAAGC  | TGTTTAAGAA  |
|      | TACCAACAAC  | AGTAACAGCC   | GCGTTGATAG    | CCATAGTTTCG | ACAAATTCTT  |
| 1501 | ATTCACCTCG  | AAAGCAAGCT   | GATAAAGGAG    | GTTTCTCGAT  | CGAGACGTTN  |
|      | TAAGTGAGAC  | TTTCGTTCTGA  | CTATTTCTCTC   | CAAAGAGCTA  | GCTCTGCAAN  |
| 1551 | NNNGAGGTTT  | CAACTTTTCACT | CATAATGAAA    | TAAGATCACT  | ACCGGGCGTA  |
|      | NNNCTCCAAG  | GTTGAAAGTG   | GTATTACTTTT   | ATTCTAGTGA  | TGGCCCCGCT  |
| 1601 | TTTTTTTGAGT | TATCGAGATT   | TTCAGGAGCT    | AAGGAAGCTA  | AAATGGAGAA  |
|      | AAAAAACTCA  | ATAGCTCTAA   | AAGTCCTCGA    | TTCCTTCGAT  | TTTACCTCTT  |
| 1651 | AAAAATCACT  | GGATATACCA   | CCGTTGATAT    | ATCCCAATGG  | CATCGTAAAG  |
|      | TTTTTTAGTGA | CCTATATGGT   | GGCAACTATA    | TAGGGTTACC  | GTAGCATTTT  |

17/39

|      |                           |                           |                           |                           |                           |
|------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| 1701 | AACATTTTGA<br>TTGTAAAACT  | GGCATTTTCAG<br>CCGTAAAGTC | TCAGTTGCTC<br>AGTCAACGAG  | AATGTACCTA<br>TTACATGGAT  | TAACCAGACC<br>ATTGGTCTGG  |
| 1751 | G TTCAGCTGG<br>CAAGTCGACC | ATATTACGGC<br>TATAATGCCG  | CTTTTTTAAAG<br>GAAAAATTTC | ACCGTAAAGA<br>TGGCATTCT   | AAAATAAGCA<br>TTTTATTCTG  |
| 1801 | CAAGTTTTAT<br>GTTCAAAATA  | CCGGCCTTTA<br>GGCCGGAAAT  | TTCACATTCT<br>AAGTGTAAGA  | TGCCCCGCTG<br>ACGGGCGGAC  | ATGAATGCTC<br>TACTTACGAG  |
| 1851 | ATCCGGAGTT<br>TAGGCCTCAA  | CCGTATGGCA<br>GGCATACCGT  | ATGAAAGACG<br>TACTTTCTGC  | GTGAGCTGGT<br>CACTCGACCA  | GATATGGGAT<br>CTATACCCTA  |
| 1901 | AGTGTTTACC<br>TCACAAGTGG  | CTTGTTACAC<br>GAACAATGTG  | CGTTTTCCAT<br>GCAAAAGGTA  | GAGCAAACCTG<br>CTCGTTTGAC | AAACGTTTTTC<br>TTTGCAAAAG |
| 1951 | ATCGCTCTGG<br>TAGCGAGACC  | AGTGAATACC<br>TCACTTATGG  | ACGACGATTT<br>TGCTGCTAAA  | CCGGCAGTTT<br>GGCCGTCAA   | CTACACATAT<br>GATGTGTATA  |
| 2001 | ATTCGCAAGA<br>TAAGCGTTCT  | TGTGGCGTGT<br>ACACCGCACA  | TACGGTGAAA<br>ATGCCACTTT  | ACCTGGCCTA<br>TGGACCGGAT  | TTTCCCTAAA<br>AAAGGGATTT  |
| 2051 | GGGTTTATTG<br>CCCAAATAAC  | AGAATATGTT<br>TCTTATACAA  | TTTCGTCTCA<br>AAAGCAGAGT  | GCCAATCCCT<br>CGGTTAGGGA  | GGGTGAGTTT<br>CCCACTCAA   |
| 2101 | CACCAGTTTT<br>GTGGTCAAAA  | GATTTAAACG<br>CTAAATTTGC  | TAGCCAATAT<br>ATCGGTTATA  | GGACAACTTC<br>CCTGTTGAAG  | TTCGCCCCCG<br>AAGCGGGGGC  |
| 2151 | TTTTCACTAT<br>AAAAGTGATA  | GGGCAAATAT<br>CCCGTTTATA  | TATACGCAAG<br>ATATGCGTTC  | GCGACAAGGT<br>CGCTGTTCCA  | GCTGATGCCG<br>CGACTACGGC  |
| 2201 | CTGGCGATTG<br>GACCGCTAAG  | AGGTTTATCA<br>TCCAAGTAGT  | TGCCGTTTGT<br>ACGGCAAACA  | GATGGCTTCC<br>CTACCGAAGG  | ATGTCGGCAG<br>TACAGCCGTC  |
| 2251 | AATGCTTAAT<br>TTACGAATTA  | GAATTACAAC<br>CTTAATGTTG  | AGTACTGCGA<br>TCATGACGCT  | TGAGTGGCAG<br>ACTCACCGTC  | GGCGGGGCGT<br>CCGCCCCGCA  |
| 2301 | AATTTTTTTA<br>TTAAAAAAT   | AGGCAGTTAT<br>TCCGTCAATA  | TGGTGCCCTT<br>ACCACGGGAA  | AAACGCCTGG<br>TTTGCGGACC  | TGCTAGCCTG<br>ACGATCGGAC  |
| 2351 | AGGCCAGTTT<br>TCCGTCAAA   | GCTCAGGCTC<br>CGAGTCCGAG  | TCCCCGTGGA<br>AGGGGCACCT  | GGTAATAATT<br>CCATTATTAA  | GCTCGACCGA<br>CGAGCTGGCT  |
| 2401 | TAAAAGCGGC<br>ATTTTCGCCG  | TTCCTGACAG<br>AAGGACTGTC  | GAGGCCGTTT<br>CTCCGGCAA   | TGTTTTGCAG<br>ACAAAACGTC  | CCCACCTCAA<br>GGGTGGAGTT  |
| 2451 | CGCAATTAAT<br>GCGTTAATTA  | GTGAGTTAGC<br>CACTCAATCG  | TCACTCATTA<br>AGTGAGTAAT  | GGCACCCCAG<br>CCGTGGGGTC  | GCTTTTAACT<br>CGAAATGTGA  |
| 2501 | TTATGCTTCC<br>AATACGAAGG  | GGCTCGTATG<br>CCGAGCATAC  | TTGTGTGGAA<br>AACACACCTT  | TTGTGAGCGG<br>AACACTCGCC  | ATAACAATTT<br>TATTGTTAAA  |

18/39

|      |             |             |            |            |            |
|------|-------------|-------------|------------|------------|------------|
| 2551 | CACACAGGAA  | ACAGCTATGA  | CCATGATTAC | GAATTTCTAG | ATAACGAGGG |
|      | GTGTGTCCTT  | TGTCGATACT  | GGTACTAATG | CTTAAAGATC | TATTGCTCCC |
| 2601 | CAAAAAATGA  | AAAAGACAGC  | TATCGCGATT | GCAGTGGCAC | TGGCTGGTTT |
|      | GTTTTTTTACT | TTTTCTGTCTG | ATAGCGCTAA | CGTCACCGTG | ACCGACCAAA |
| 2651 | CGCTACCGTA  | GCGCAGGCCG  | ACTACAAAGA | TGTCGACGCC | GGTGGTCGGA |
|      | GCGATGGCAT  | CGCGTCCGGC  | TGATGTTTCT | ACAGCTGCGG | CCACCAGCCT |
| 2701 | TCGCCCCGGCT | AGAGGAAAAA  | GTGAAAACCT | TGAAAGCGCA | AAACTCCGAG |
|      | AGCGGGCCGA  | TCTCCTTTTT  | CACTTTTTGA | ACTTTCGCGT | TTTGAGGCTC |
| 2751 | CTGGCGTCCA  | CGGCCAACAT  | GCTCAGGGAA | CAGGTGGCAC | AGCTTAAACA |
|      | GACCGCAGGT  | GCCGGTTGTA  | CGAGTCCCTT | GTCCACCGTG | TCGAATTTGT |

EcoRI

~~~~~

2801	GAAAGTCATG	AACCACGGTG	GTGCCGAATT	CAATGCTGGC	GGCGGCTCTG
	CTTTCAGTAC	TTGGTGCCAC	CACGGCTTAA	GTTACGACCG	CCGCCGAGAC
2851	GTGGTGGTTC	TGGTGGCGGC	TCTGAGGGTG	GTGGCTCTGA	GGGTGGCGGT
	CACCACCAAG	ACCACCGCCG	AGACTCCAC	CACCGAGACT	CCCACCGCCA
2901	TCTGAGGGTG	GCGGCTCTGA	GGGAGGCGGT	TCCGGTGGTG	GCTCTGGTTC
	AGACTCCAC	CGCCGAGACT	CCCTCCGCCA	AGGCCACCAC	CGAGACCAAG
2951	CGGTGATTTT	GATTATGAAA	AGATGGCAAA	CGCTAATAAG	GGGGCTATGA
	GCCACTAAAA	CTAATACTTT	TCTACCGTTT	GCGATTATTC	CCCCGATACT
3001	CCGAAAATGC	CGATGAAAAC	GCGCTACAGT	CTGACGCTAA	AGGCAAACCT
	GGCTTTTACG	GCTACTTTTG	CGCGATGTCA	GACTGCGATT	TCCGTTTGAA

ClaI

~~~~~

|      |            |            |            |            |             |
|------|------------|------------|------------|------------|-------------|
| 3051 | GATTCTGTCG | CTACTGATTA | CGGTGCTGCT | ATCGATGGTT | TCATTGGTGA  |
|      | CTAAGACAGC | GATGACTAAT | GCCACGACGA | TAGCTACCAA | AGTAACCACT  |
| 3101 | CGTTTCCGGC | CTTGCTAATG | GTAATGGTGC | TACTGGTGAT | TTTGCTGGCT  |
|      | GCAAAGGCCG | GAACGATTAC | CATTACCACG | ATGACCACTA | AAACGACCGA  |
| 3151 | CTAATTCCCA | AATGGCTCAA | GTCGGTGACG | GTGATAATTC | ACCTTTAATG  |
|      | GATTAAGGGT | TTACCGAGTT | CAGCCACTGC | CACTATTAAG | TGGAAATTAC  |
| 3201 | AATAATTTCC | GTCAATATTT | ACCTTCCCTC | CCTCAATCGG | TTGAATGTCTG |
|      | TTATTAAAGG | CAGTTATAAA | TGGAAGGGAG | GGAGTTAGCC | AACTTACAGC  |
| 3251 | CCCTTTTGTC | TTTAGCGCTG | GTAAACCATA | TGAATTTTCT | ATTGATTGTG  |
|      | GGGAAAACAG | AAATCGCGAC | CATTTGGTAT | ACTTAAAAGA | TAATAACAC   |
| 3301 | ACAAAATAAA | CTTATTCCGT | GGTGTCTTTG | CGTTTCTTTT | ATATGTTGCC  |
|      | TGTTTTATTT | GAATAAGGCA | CCACAGAAAC | GCAAAGAAAA | TATACAACGG  |

19/39

3351 ACCTTTATGT ATGTATTTTC TACGTTTGCT AACATACTGC GTAATAAGGA  
TGGAATACA TACATAAAAG ATGCAAACGA TTGTATGACG CATTATTCCT

HindIII

~~~~~

3401 GTCTTGATAA GCTTCGAGAA ATTCACCTCG AAAGCAAGCT GATAAACCGA  
CAGAACTATT CGAAGCTCTT TAAGTGAGAC TTTCGTTCTGA CTATTTGGCT

3451 TACAATTAAA GGCTCCTTTT GGAGCCTTTT TTTTGGAGA ATTAATTCAA  
ATGTTAATTT CCGAGGAAAA CCTCGGAAAA AAAACCTCT TAATTAAGTT

3501 TCATGCCAGT TCTTTTGGGT ATTCCGTTAT TATTGCGTTT CCTCGGTTTC  
AGTACGGTCA AGAAAACCCA TAAGGCAATA ATAACGCAA GGAGCCAAAG

3551 CTTCTGGTAA CTTTGTTTCGG CTATCTGCTT ACTTTCCTTA AAAAGGGCTT  
GAAGACCATT GAAACAAGCC GATAGACGAA TGAAAGGAAT TTTTCCCGAA

3601 CGGTAAGATA GCTATTGCTA TTTCATTGTT TCTTGCTCTT ATTATTGGGC  
GCCATTCTAT CGATAACGAT AAAGTAACAA AGAACGAGAA TAATAACCCG

3651 TTAAGTCAAT TCTTGTGGGT TATCTCTCTG ATATTAGCGC ACAATTACCC  
AATTGAGTTA AGAACACCCA ATAGAGAGAC TATAATCGCG TGTTAATGGG

3701 TCTGATTTTG TTCAGGGCGT TCAGTTAATT CTCCCGTCTA ATGCGCTTCC  
AGACTAAAAC AAGTCCCGCA AGTCAATTAA GAGGGCAGAT TACGCGAAGG

3751 CTGTTTTTAT GTTATTCTCT CTGTAAAGGC TGCTATTTTC ATTTTGGACG  
GACAAAAATA CAATAAGAGA GACATTTCAG ACGATAAAAG TAAAACTGC

3801 TTAAACAAAA AATCGTTTCT TATTTGGATT GGGATAAATA AATATGGCTG  
AATTTGTTTT TTAGCAAAGA ATAAACCTAA CCCTATTTAT TTATACCGAC

3851 TTTATTTTGT AACTGGCAAA TTAGGCTCTG GAAAGACGCT CGTTAGCGTT  
AAATAAAACA TTGACCGTTT AATCCGAGAC CTTTCTGCGA GCAATCGCAA

3901 GGTAAGATTC AGGATAAAAT TGTAGCTGGG TGCAAAATAG CAACTAATCT  
CCATTCTAAG TCCTATTTTA ACATCGACCC ACGTTTTATC GTTGATTAGA

3951 TGATTTAAGG CTTCAAAACC TCCCGCAAGT CGGGAGGTTT GCTAAAACGC  
ACTAAATTCC GAAGTTTGG AGGGCGTTCA GCCCTCCAAG CGATTTTGCG

4001 CTCGCGTTCT TAGAATACCG GATAAGCCTT CTATTTCTGA TTTGCTTGCT  
GAGCGCAAGA ATCTTATGGC CTATTCGGAA GATAAAGACT AAACGAACGA

4051 ATTGGTCGTG GTAATGATTC CTACGACGAA AATAAAAACG GTTTGCTTGT  
TAACCAGCAC CATTACTAAG GATGCTGCTT TTATTTTTCG CAAACGAACA

4101 TCTTGATGAA TGCGGTACTT GGTTTAATAC CCGTTCATGG AATGACAAGG  
AGAAGTACTT ACGCCATGAA CCAAATTATG GGCAAGTACC TTACTGTTCC



20/39

4151	AAAGACAGCC	GATTATTGAT	TGGTTTCTTC	ATGCTCGTAA	ATTGGGATGG
	TTTCTGTCGG	CTAATAACTA	ACCAAAGAAG	TACGAGCATT	TAACCCTACC
4201	GATATTATTT	TTCTTGTTCA	GGATTTATCT	ATTGTTGATA	AACAGGCGCG
	CTATAATAAA	AAGAACAAGT	CCTAAATAGA	TAACAACAT	TTGTCCGCGC
4251	TTCTGCATTA	GCTGAACACG	TTGTTTATTG	TCGCCGTCTG	GACAGAATTA
	AAGACGTAAT	CGACTTGTGC	AACAAATAAC	AGCGGCAGAC	CTGTCTTAAT
4301	CTTTACCCTT	TGTCGGCACT	TTATATTCTC	TTGTTACTGG	CTCAAAAATG
	GAAATGGGAA	ACAGCCGTGA	AATATAAGAG	AACAATGACC	GAGTTTTTAC
4351	CCTCTGCCTA	AATTACATGT	TGGTGTGTGT	AAATATGGTG	ATTCTCAATT
	GGAGACGGAT	TTAATGTACA	ACCACAACAA	TTTATACCAC	TAAGAGTTAA
4401	AAGCCCTACT	GTTGAGCGTT	GGCTTTATAC	TGGTAAGAAT	TTATATAACG
	TTCGGGATGA	CAACTCGCAA	CCGAAATATG	ACCATTCTTA	AATATATTGC
4451	CATATGACAC	TAAACAGGCT	TTTTCCAGTA	ATTATGATTC	AGGTGTTTAT
	GTATACTGTG	ATTTGTCCGA	AAAAGGTCAT	TAATACTAAG	TCCACAAATA
4501	TCATATTTAA	CCCCTTATTT	ATCACACGGT	CGGTATTTCA	AACCATTAAA
	AGTATAAATT	GGGGAATAAA	TAGTGTGCCA	GCCATAAAGT	TTGGTAATTT
4551	TTTAGGTCAG	AAGATGAAAT	TAACTAAAAT	ATATTTGAAA	AAGTTTTCTC
	AAATCCAGTC	TTCTACTTTA	ATTGATTTTA	TATAAACTTT	TTCAAAAGAG
4601	GCGTTCTTTG	TCTTGCGATA	GGATTTGCAT	CAGCATTTAC	ATATAGTTAT
	CGCAAGAAAC	AGAACGCTAT	CCTAAACGTA	GTCGTAAATG	TATATCAATA
4651	ATAACCCAAC	CTAAGCCGGA	GGTTAAAAAG	GTAGTCTCTC	AGACCTATGA
	TATTGGGTTG	GATTCGGCCT	CCAATTTTTC	CATCAGAGAG	TCTGGATACT
4701	TTTTGATAAA	TTCACTATTG	ACTCTTCTCA	GCGTCTTAAT	CTAAGCTATC
	AAAACATTTT	AAGTGATAAC	TGAGAAGAGT	CGCAGAATTA	GATTCGATAG
4751	GCTATGTTTT	CAAGGATTCT	AAGGGAAAAT	TAATTAATAG	CGACGATTTA
	CGATACAAAA	GTTCCCTAAGA	TTCCCTTTTA	ATTAATTATC	GCTGCTAAAT
4801	CAGAAGCAAG	GTTATTCCAT	CACATATATT	GATTTATGTA	CTGTTTCAAT
	GTCTTCGTTC	CAATAAGGTA	GTGTATATAA	CTAAATACAT	GACAAAGTTA
4851	TAAAAAAGGT	AATTCAAATG	AAATTGTTAA	ATGTAATTAA	TTTTGTTTTC
	ATTTTTTCCA	TTAAGTTTAC	TTTAACAATT	TACATTAATT	AAAACAAAAG
4901	TTGATGTTTG	TTTCATCATC	TTCTTTTGCT	CAAGTAATTG	AAATGAATAA
	AACTACAAAC	AAAGTAGTAG	AAGAAAACGA	GTTCAATTAAC	TTTACTTATT
4951	TTCGCCTCTG	CGCGATTTTCG	TGACTTGGTA	TTCAAAGCAA	ACAGGTGAAT
	AAGCGGAGAC	GCGCTAAAGC	ACTGAACCAT	AAGTTTCGTT	TGTCCACTTA

21/39

5001	CTGTTATTGT	CTCACCTGAT	GTTAAAGGTA	CAGTGACTGT	ATATTCCTCT
	GACAATAACA	GAGTGGACTA	CAATTTCCAT	GTCAC TGACA	TATAAGGAGA
5051	GACGTTAAGC	CTGAAAATTT	ACGCAATTTT	TTTATCTCTG	TTTTACGTGC
	CTGCAATTCG	GACTTTTAAA	TGCGTTAAAG	AAATAGAGAC	AAAATGCACG
5101	TAATAATTTT	GATATGGTTG	GCTCAATTCC	TTCCATAATT	CAGAAATATA
	ATTATTAAAA	CTATACCAAC	CGAGTTAAGG	AAGGTATTAA	GTCTTTATAT
5151	ACCCAAATAG	TCAGGATTAT	ATTGATGAAT	TGCCATCATC	TGATATTCAG
	TGGGTTTATC	AGTCCTAATA	TAAC TACTTA	ACGGTAGTAG	ACTATAAGTC
5201	GAATATGATG	ATAATTCCGC	TCCTTCTGGT	GGTTTCTTTG	TTCCGCAAAA
	CTTATACTAC	TATTAAGGCG	AGGAAGACCA	CCAAAGAAAC	AAGGCGTTTT
5251	TGATAATGTT	ACTCAAACAT	TTAAAATTAA	TAACGTTTCG	GCAAAGGATT
	ACTATTACAA	TGAGTTTGTA	AATTTTAATT	ATTGCAAGCG	CGTTTCCTAA
5301	TAATAAGGGT	TGTAGAATTG	TTTGTTAAAT	CTAATACATC	TAAATCCTCA
	ATTATTCCCA	ACATCTTAAC	AAACAATTTA	GATTATGTAG	ATTTAGGAGT
5351	AATGTATTAT	CTGTTGATGG	TTCTAACTTA	TTAGTAGTTA	GCGCCCCTAA
	TTACATAATA	GACA ACTACC	AAGATTGAAT	AATCATCAAT	CGCGGGGATT
5401	AGATATTTTA	GATAACCTTC	CGCAATTTCT	TTCTACTGTT	GATTTGCCAA
	TCTATAAAAT	CTATTGGAAG	GCGTTAAAGA	AAGATGACAA	CTAAACGGTT
5451	CTGACCAGAT	ATTGATTGAA	GGATTAATTT	TCGAGGTTCA	GCAAGGTGAT
	GACTGGTCTA	TAAC TAACTT	CCTAATTAAA	AGCTCCAAGT	CGTTCCACTA
5501	GCTTTAGATT	TTTCCTTTGC	TGCTGGCTCT	CAGCGCGGCA	CTGTTGCTGG
	CGAAATCTAA	AAAGGAAACG	ACGACCGAGA	GTCGCGCCGT	GACAACGACC
5551	TGGTGTTAAT	ACTGACCGTC	TAACCTCTGT	TTTATCTTCT	GCGGGTG GTT
	ACCACAATTA	TGACTGGCAG	ATTGGAGACA	AAATAGAAGA	CGCCCACCAA
5601	CGTTCGGTAT	TTTTAACGGC	GATGTTTTAG	GGCTATCAGT	TCGCGCATTA
	GCAAGCCATA	AAAATTGCCG	CTACAAAATC	CCGATAGTCA	AGCGCGTAAT
5651	AAGACTAATA	GCCATTCAAA	AATATTGTCT	GTGCCTCGTA	TTCTTACGCT
	TTCTGATTAT	CGGTAAGTTT	TTATAACAGA	CACGGAGCAT	AAGAATGCGA
5701	TTCAGGTCAG	AAGGGTTCTA	TTTCTGTTGG	CCAGAA TGTC	CCTTTTATTA
	AAGTCCAGTC	TTCCCAAGAT	AAAGACAACC	GGTCTTACAG	GGAAAATAAT
5751	CTGGTCGTGT	AACTGGTGAA	TCTGCCAATG	TAAATAATCC	ATTTCAGACG
	GACCAGCACA	TTGACCACTT	AGACGGTTAC	ATTTATTAGG	TAAAGTCTGC
5801	GTTGAGCGTC	AAAATGTTGG	TATTTCTATG	AGTGTTTTTC	CCGTTGCAAT
	CAACTCGCAG	TTTTACAACC	ATAAAGATAC	TCACAAAAAG	GGCAACGTTA

22/39

5851	GGCTGGCGGT	AATATTGTTT	TAGATATAAC	CAGTAAGGCC	GATAGTTTGA
	CCGACCGCCA	TTATAACAAA	ATCTATATTG	GTCATTCCGG	CTATCAAAC
5901	GTTCTTCTAC	TCAGGCAAGT	GATGTTATTA	CTAATCAAAG	AAGTATTGCG
	CAAGAAGATG	AGTCCGTTCA	CTACAATAAT	GATTAGTTTC	TTCATAACGC
5951	ACAACGGTTA	ATTTGCGTGA	TGGTCAGACT	CTTTTGCTCG	GTGGCCTCAC
	TGTTGCCAAT	TAAACGCACT	ACCAGTCTGA	GAAAACGAGC	CACCGGAGTG
6001	TGATTACAAA	AACACTTCTC	AAGATTCTGG	TGTGCCGTTC	CTGTCTAAAA
	ACTAATGTTT	TTGTGAAGAG	TTCTAAGACC	ACACGGCAAG	GACAGATTTT
6051	TCCCTTTAAT	CGGCCTCCTG	TTTAGCTCCC	GTTCTGATTG	TAACGAGGAA
	AGGGAAATTA	GCCGGAGGAC	AAATCGAGGG	CAAGACTAAG	ATTGCTCCTT
6101	AGCACGTTGT	ACGTGCTCGT	CAAAGCAACC	ATAGTACGCG	CCCTGTAGCG
	TCGTGCAACA	TGCACGAGCA	GTTTCGTTGG	TATCATGCGC	GGGACATCGC
6151	GCGCATTAAAG	CGCGGCGGGT	GTGGTGGTTA	CGCGCAGCGT	GACCGCTACA
	CGCGTAATTC	GCGCCGCCCA	CACCACCAAT	GCGCGTCGCA	CTGGCGATGT
6201	CTTGCCAGCG	CCCTAGCGCC	CGCTCCTTTC	GCTTTCTTCC	CTTCCTTTCT
	GAACGGTCGC	GGGATCGCGG	GCGAGGAAAG	CGAAAGAAGG	GAAGGAAAGA
				BamHI	
				~~~~~	
6251	CGCCACGTTC	TCCGGCTTTC	CCCGTCAAGC	TCTAAATCGG	GGGATCCCTT
	GCGGTGCAAG	AGGCCGAAAG	GGGCAGTTTC	AGATTTAGCC	CCCTAGGGAA
6301	TAGGGTTCCG	ATTTAGTGCT	TTACGGCACC	TCGACCTCCA	AAAACCTTGAT
	ATCCCAAGGC	TAAATCACGA	AATGCCGTGG	AGCTGGAGGT	TTTTGAACTA
6351	TTGGGTGATG	GTTACGCTAG	TGGGCCATCG	CCCTGATAGA	CGGTTTTTTCG
	AACCCACTAC	CAAGTGCATC	ACCCGGTAGC	GGGACTATCT	GCCAAAAAGC
6401	CCCTTTGACG	TTGGAGTCCA	CGTTCTTTAA	TAGTGGACTC	TTGTTCCAAA
	GGGAAACTGC	AACCTCAGGT	GCAAGAAATT	ATCACCTGAG	AACAAGGTTT
6451	CTGGAACAAC	ACTCACAAC	AATCGGCCT	ATTCTTTTGA	TTTATAAGGA
	GACCTTGTTG	TGAGTGTTGA	TTGAGCCGGA	TAAGAAAAC	AAATATTCTT
6501	TTTTTGTCAT	TTTCTGCTTA	CTGGTTAAAA	AATAAGCTGA	TTTAACAAAT
	AAAAACAGTA	AAAGACGAAT	GACCAATTTT	TTATTGACT	AAATTGTTTA
6551	ATTTAACGCG	AAATTTAACA	AAACATTAAC	GTTTACAATT	TAAATATTTG
	TAAATTGCGC	TTTAAATTGT	TTTGTAATTG	CAAATGTAA	ATTTATAAAC
6601	CTTATACAAT	CATCCTGTTT	TTGGGGCTTT	TCTGATTATC	AACCGGGGTA
	GAATATGTTA	GTAGGACAAA	AACCCCGAAA	AGACTAATAG	TTGGCCCCAT

23/39

Clai

~~~~~

6651 CATATGATTG ACATGCTAGT TTTACGATTA CCGTTCATCG ATTCTCTTGT  
GTATACTAAC TGTACGATCA AAATGCTAAT GGCAAGTAGC TAAGAGAACA

6701 TTGCTCCAGA CTTTCAGGTA ATGACCTGAT AGCCTTTGTA GACCTCTCAA  
AACGAGGTCT GAAAGTCCAT TACTGGACTA TCGGAAACAT CTGGAGAGTT

6751 AAATAGCTAC CCTCTCCGGC ATGAATTTAT CAGCTAGAAC GGTTGAATAT  
TTTATCGATG GGAGAGGCCG TACTTAAATA GTCGATCTTG CCAACTTATA

6801 CATATTGACG GTGATTTGAC TGTCTCCGGC CTTTCTCACC CGTTTGAATC  
GTATAACTGC CACTAAACTG ACAGAGGCCG GAAAGAGTGG GCAAACTTAG

6851 TTTGCCTACT CATTACTCCG GCATTGCATT TAAAATATAT GAGGGTTCTA  
AAACGGATGA GTAATGAGGC CGTAACGTAA ATTTTATATA CTCCCAAGAT

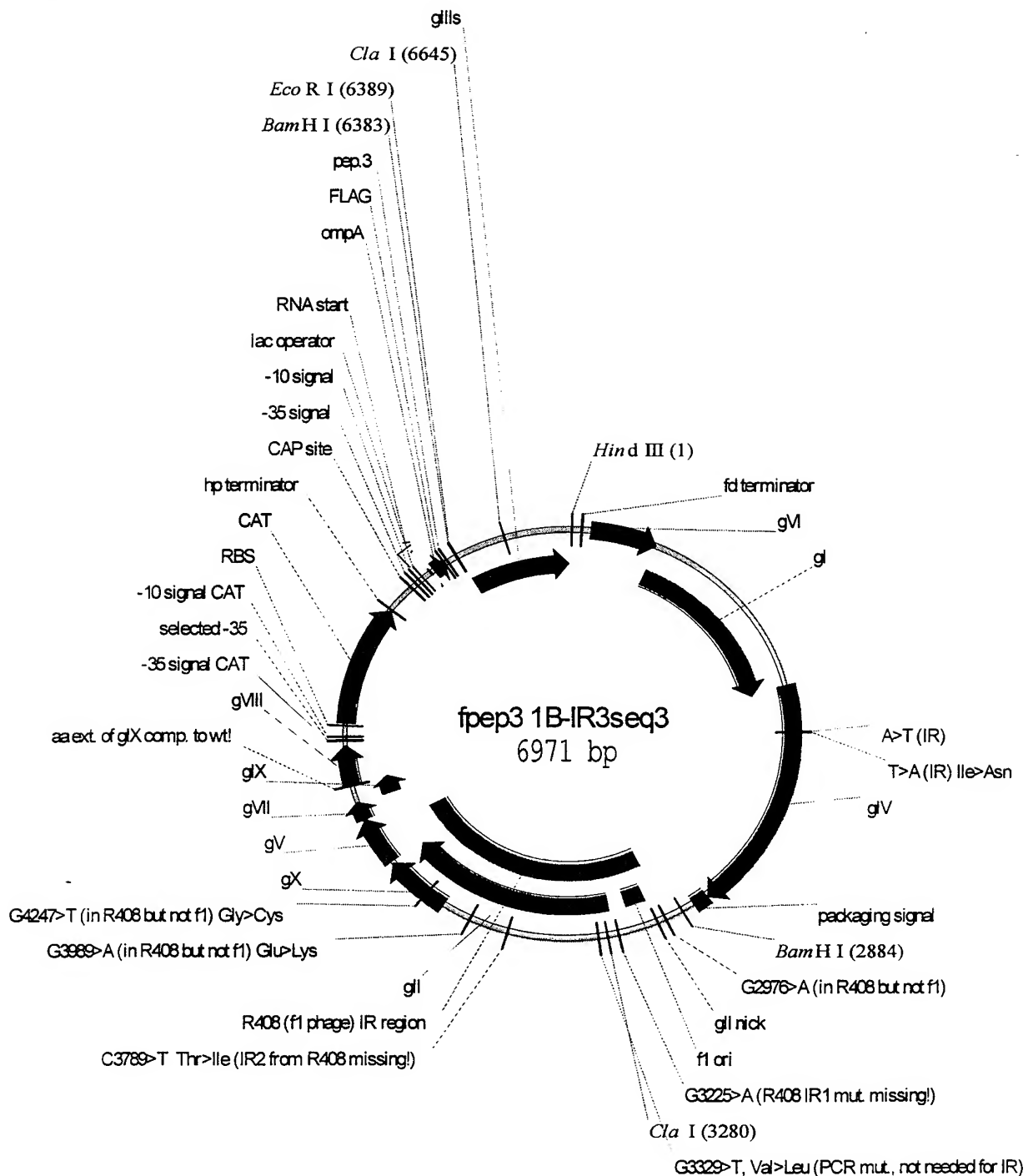
6901 AAAATTTTTTA TCCCTGCGTT GAAATTAAGG CTTCAACCAGC AAAAGTATTA  
TTTTAAAAAT AGGGACGCAA CTTTAATTCC GAAGTGGTCG TTTTCATAAT

6951 CAGGGTCATA ATGTTTTTGG TACAACCGAT TTAGCTTTAT GCTCTGAGGC  
GTCCCAGTAT TACAAAAACC ATGTTGGCTA AATCGAAATA CGAGACTCCG

7001 TTTATTGCTT AATTTTGCTA ACTCTCTGCC TTGCTTGTAC GATTTATTGG  
AAATAACGAA TTAAAACGAT TGAGAGACGG AACGAACATG CTAAATAACC

7051 ATGTT  
TACAA

24/39

**Figure 4**

25/39

HindIII

~~~~~

```

1  AGCTTCGAGA AATTCACCTC GAAAGCAAGC TGATAAACCG ATACAATTAA
   TCGAAGCTCT TTAAGTGGAG CTTTCGTTTCG ACTATTTGGC TATGTTAATT

51  AGGCTCCTTT TGGAGCCTTT TTTTTTGGAG AATTAATTCA ATCATGCCAG
   TCCGAGGAAA ACCTCGGAAA AAAAAACCTC TTAATTAAGT TAGTACGGTC

101 TTCTTTTGGG TATTCCGTTA TTATTGCGTT TCCTCGGTTT CTTTCTGGTA
   AAGAAAACCC ATAAGGCAAT AATAACGCAA AGGAGCCAAA GGAAGACCAT

151 ACTTTGTTCG GCTATCTGCT TACTTTCCTT AAAAAGGGCT TCGGTAAGAT
   TGAAACAAGC CGATAGACGA ATGAAAGGAA TTTTTCCTCGA AGCCATTCTA

201 AGCTATTGCT ATTTCATTGT TTCTTGCTCT TATTATTGGG CTTAACTCAA
   TCGATAACGA TAAAGTAACA AAGAACGAGA ATAATAACCC GAATTGAGTT

251 TTCTTGTGGG TTATCTCTCT GATATTAGCG CACAATTACC CTCTGATTTT
   AAGAACACCC AATAGAGAGA CTATAATCGC GTGTTAATGG GAGACTAAAA

301 GTTCAGGGCG TTCAGTTAAT TCTCCCGTCT AATGCGCTTC CCTGTTTTTA
   CAAGTCCCGC AAGTCAATTA AGAGGGCAGA TTACGCGAAG GGACAAAAAT

351 TGTTATTCTC TCTGTAAAGG CTGCTATTTT CATTTTTGAC GTTAAACAAA
   ACAATAAGAG AGACATTTCC GACGATAAAA GTAAAAACTG CAATTTGTTT

401 AAATCGTTTC TTATTTGGAT TGGGATAAAT AAATATGGCT GTTTATTTTG
   TTTAGCAAAG AATAAACCTA ACCCTATTTA TTTATACCGA CAAATAAAAC

451 TAACTGGCAA ATTAGGCTCT GGAAAGACGC TCGTTAGCGT TGGTAAGATT
   ATTGACCGTT TAATCCGAGA CCTTCTGCG AGCAATCGCA ACCATTCTAA

501 CAGGATAAAA TTGTAGCTGG GTGCAAAATA GCAACTAATC TTGATTTAAG
   GTCCTATTTT AACATCGACC CACGTTTTAT CGTTGATTAG AACTAAATTC

551 GCTTCAAAAC CTCCCGCAAG TCGGGAGGTT CGCTAAACG CCTCGCGTTC
   CGAAGTTTTG GAGGGCGTTC AGCCCTCCAA GCGATTTTGC GGAGCGCAAG

601 TTAGAATACC GGATAAGCCT TCTATTTCTG ATTTGCTTGC TATTGGTCGT
   AATCTTATGG CCTATTCGGA AGATAAAGAC TAAACGAACG ATAACCAGCA

651 GGTAATGATT CCTACGACGA AAATAAAAAC GGTTTGCTTG TTCTTGATGA
   CCATTACTAA GGATGCTGCT TTTATTTTTG CCAAACGAAC AAGAACTACT

701 ATGCGGTACT TGGTTTAATA CCCGTTTCATG GAATGACAAG GAAAGACAGC
   TACGCCATGA ACCAAATTAT GGGCAAGTAC CTTACTGTTC CTTTCTGTGC

751 CGATTATTGA TTGGTTTCTT CATGCTCGTA AATTGGGATG GGATATTATT
   GCTAATAACT AACCAGAGAA GTACGAGCAT TTAACCCTAC CCTATAATAA

```

26/39

801	TTTCTTGTTT	AGGATTTATC	TATTGTTGAT	AAACAGGCGC	GTTCTGCATT
	AAAGAACAAG	TCCTAAATAG	ATAACAATA	TTTGTCCGCG	CAAGACGTAA
851	AGCTGAACAC	GTTGTTTATT	GTCGCCGTCT	GGACAGAAAT	ACTTTACCCT
	TCGACTTGTG	CAACAAATAA	CAGCGGCAGA	CCTGTCTTAA	TGAAATGGGA
901	TTGTCCGCAC	TTTATATTCT	CTTGTTACTG	GCTCAAAAAT	GCCTCTGCCT
	AACAGCCGTG	AAATATAAGA	GAACAATGAC	CGAGTTTTTA	CGGAGACGGA
951	AAATTACATG	TTGGTGTGTG	TAAATATGGT	GATTCTCAAT	TAAGCCCTAC
	TTTAATGTAC	AACCACAACA	ATTTATACCA	CTAAGAGTTA	ATTCGGGATG
1001	TGTTGAGCGT	TGGCTTTATA	CTGGTAAGAA	TTTATATAAC	GCATATGACA
	ACAACCTCGA	ACCGAAATAT	GACCATTCTT	AAATATATTG	CGTATACTGT
1051	CTAAACAGGC	TTTTTCCAGT	AATTATGATT	CAGGTGTTTA	TTCATATTTA
	GATTTGTCCG	AAAAAGGTCA	TTAATACTAA	GTCCACAAAT	AAGTATAAAT
1101	ACCCCTTATT	TATCACACGG	TCGGTATTTT	AAACCATTAA	ATTTAGGTCA
	TGGGGAATAA	ATAGTGTGCC	AGCCATAAAG	TTTGGTAATT	TAAATCCAGT
1151	GAAGATGAAA	TTAACTAAAA	TATATTTGAA	AAAGTTTTCT	CGCGTTCTTT
	CTTCTACTTT	AATTGATTTT	ATATAAACTT	TTTCAAAAGA	GCGCAAGAAA
1201	GTCTTGCGAT	AGGATTTGCA	TCAGCATTTA	CATATAGTTA	TATAACCCAA
	CAGAACGCTA	TCCTAAACGT	AGTCGTAAAT	GTATATCAAT	ATATTGGGTT
1251	CCTAAGCCGG	AGGTTAAAAA	GGTAGTCTCT	CAGACCTATG	ATTTTGATAA
	GGATTCCGGC	TCCAATTTTT	CCATCAGAGA	GTCTGGATAC	TAAAACCTAT
1301	ATTCACTATT	GACTCTTCTC	AGCGTCTTAA	TCTAAGCTAT	CGCTATGTTT
	TAAGTGATAA	CTGAGAAGAG	TCGCAGAATT	AGATTTCGATA	GCGATACAAA
1351	TCAAGGATTC	TAAGGGAAAA	TTAATTAATA	GCGACGATTT	ACAGAAGCAA
	AGTTCCTAAG	ATTCCCTTTT	AATTAATTAT	CGCTGCTAAA	TGTCTTCGTT
1401	GGTTATTCCA	TCACATATAT	TGATTTATGT	ACTGTTTCAA	TTAAAAAAGG
	CCAATAAGGT	AGTGTATATA	ACTAAATACA	TGACAAAGTT	AATTTTTTCC
1451	TAATTCAAAT	GAAATTGTTA	AATGTAATTA	ATTTTGTTTT	CTTGATGTTT
	ATTAAGTTTA	CTTTAACAAT	TTACATTAAT	TAAAACAAAA	GAAC TACAAA
1501	GTTTCATCAT	CTTCTTTTGC	TCAAGTAATT	GAAATGAATA	ATTCGCCTCT
	CAAAGTAGTA	GAAGAAAACG	AGTTCATTAA	CTTTACTTAT	TAAGCGGAGA
1551	GCGCGATTTT	GTGACTTGGT	ATTCAAAGCA	AACAGGTGAA	TCTGTTATTG
	CGCGCTAAAG	CACTGAACCA	TAAGTTTCGT	TTGTCCACTT	AGACAATAAC
1601	TCTCACCTGA	TGTTAAAGGT	ACAGTGACTG	TATATTCCTC	TGACGTTAAG
	AGAGTGGAAT	ACAATTTCCA	TGTCCTGAC	ATATAAGGAG	ACTGCAATTC

27/39

1651	CCTGAAAATT GGACTTTTAA	TACGCAATTT ATGCGTTAAA	CTTTATCTCT GAAATAGAGA	GTTTTACGTG CAAAATGCAC	CTAATAATTT GATTATTAAA
1701	TGATATGGTT ACTATACCAA	GGCTCTAATC CCGAGATTAG	CTTCCATAAT GAAGGTATTA	TCAGAAATAT AGTCTTTATA	AACCCAAATA TTGGGTTTAT
1751	GTCAGGATTA CAGTCCTAAT	TATTGATGAA ATAACTACTT	TTGCCATCAT AACGGTAGTA	CTGATATTCA GACTATAAGT	GGAATATGAT CCTTATACTA
1801	GATAATTCCG CTATTAAGGC	CTCCTTCTGG GAGGAAGACC	TGGTTTCTTT ACCAAAGAAA	GTTCCGCAAA CAAGGCGTTT	ATGATAATGT TACTATTACA
1851	TACTCAAACA ATGAGTTTGT	TTTAAAATTA AAATTTTAAT	ATAACGTTCT TATTGCAAGC	CGCAAAGGAT GCGTTTCCTA	TTAATAAGGG AATTATTCCC
1901	TTGTAGAATT AACATCTTAA	GTTTGTTAAA CAAACAATTT	TCTAATACAT AGATTATGTA	CTAAATCCTC GATTTAGGAG	AAATGTATTA TTTACATAAT
1951	TCTGTTGATG AGACAACCTAC	GTTCTAACTT CAAGATTGAA	ATTAGTAGTT TAATCATCAA	AGCGCCCCTA TCGCGGGGAT	AAGATATTTT TTCTATAAAA
2001	AGATAACCTT TCTATTGGAA	CCGCAATTTT GGCGTTAAAG	TTTCTACTGT AAAGATGACA	TGATTTGCCA ACTAAACGGT	ACTGACCAGA TGACTGGTCT
2051	TATTGATTGA ATAACTAACT	AGGATTAATT TCCTAATTAA	TTGAGGTTTC AAGCTCCAAG	AGCAAGGTGA TCGTTCCACT	TGCTTTAGAT ACGAAATCTA
2101	TTTTCTTTTG AAAAGGAAAC	CTGCTGGCTC GACGACCGAG	TCAGCGCGGC AGTCGCGCCG	ACTGTTGCTG TGACAACGAC	GTGGTGTTAA CACCACAATT
2151	TACTGACCGT ATGACTGGCA	CTAACCTCTG GATTGGAGAC	TTTTATCTTC AAAATAGAAG	TGCGGGTGGT ACGCCCACCA	TCGTTGCGTA AGCAAGCCAT
2201	TTTTTAACGG AAAAATTGCC	CGATGTTTTA GCTACAAAAT	GGGCTATCAG CCCGATAGTC	TTGCGCGATT AAGCGCGTAA	AAAGACTAAT TTTCTGATTA
2251	AGCCATTCAA TCGGTAAGTT	AAATATTGTC TTTATAACAG	TGTGCCTCGT ACACGGAGCA	ATTCTTACGC TAAGAATGCG	TTTCAGGTCA AAAGTCCAGT
2301	GAAGGGTTCT CTTCCCAAGA	ATTTCTGTTG TAAAGACAAC	GCCAGAATGT CGGTCTTACA	CCCTTTTATT GGGAAAATAA	ACTGGTCGTG TGACCAGCAC
2351	TAAGTGGTGA ATTGACCACT	ATCTGCCAAT TAGACGGTTA	GTAAATAATC CATTTATTAG	CATTTGAGAC GTAAAGTCTG	AATTGAGCGT TTAACTCGCA
2401	CAAAATGTTG GTTTTACAAC	GTATTTCTAT CATAAAGATA	GAGTGTTTTT CTCACAAAAA	CCCGTTGCAA GGGCAACGTT	TGGCTGGCGG ACCGACCGCC
2451	TAATATTGTT ATTATAACAA	TTAGATATAA AATCTATATT	CCAGTAAGGC GGTCATTCCG	CGATAGTTTG GCTATCAAAC	AGTTCTTCTA TCAAGAAGAT



28/39

2501 CTCAGGCAAG TGATGTTATT ACTAATCAAA GAAGTATTGC GACAACGGTT  
GAGTCCGTTT ACTACAATAA TGATTAGTTT CTTCATAACG CTGTTGCCAA

2551 AATTTGCGTG ATGGTCAGAC TCTTTTGCTC GGTGGCCTCA CTGATTACAA  
TTAAACGCAC TACCAGTCTG AGAAAACGAG CCACCGGAGT GACTAATGTT

2601 AAACACTTCT CAAGATTCTG GTGTGCCGTT CCTGTCTAAA ATCCCTTTAA  
TTTGTGAAGA GTTCTAAGAC CACACGGCAA GGACAGATTT TAGGGAAATT

2651 TCGGCCTCCT GTTTAGCTCC CGTTCTGATT CTAACGAGGA AAGCACGTTG  
AGCCGGAGGA CAAATCGAGG GCAAGACTAA GATTGCTCCT TTCGTGCAAC

2701 TACGTGCTCG TCAAAGCAAC CATAGTACGC GCCCTGTAGC GGCGCATTAA  
ATGCACGAGC AGTTTCGTTG GTATCATGCG CGGGACATCG CCGCGTAATT

2751 GCGCGGCGGG TGTGGTGGTT ACGCGCAGCG TGACCGCTAC ACTTGCCAGC  
CGCGCCGCCC ACACCACCAA TGC GCGTCG C ACTGGCGATG TGAACGGTCG

2801 GCCCTAGCGC CCGCTCCTTT CGCTTTCTTC CCTTCCTTTC TCGCCACGTT  
CGGGATCGCG GGC GAGGAAA GCGAAAGAAG GGAAGGAAAG AGCGGTGCAA

BamHI

~~~~~

2851 CTCCGGCTTT CCCCGTCAAG CTCTAAATCG GGGGATCCCT TTAGGGTTCC  
GAGGCCGAAA GGGGCAGTTC GAGATTTAGC CCCCTAGGGA AATCCCAAGG

2901 GATTTAGTGC TTTACGGCAC CTCGACCTCC AAAA ACTTGA TTTGGGTGAT  
CTAAATCACG AAATGCCGTG GAGCTGGAGG TTTT TGA ACT AAACCCACTA

2951 GGTTACGTA GTGGGCCATC GCCCTAATAG ACGGTTTTTTC GCCCTTTGAC  
CCAAGTGCAT CACCCGGTAG CGGGATTATC TGCCAAAAAG CGGGAAACTG

3001 GTTGGAGTCC ACGTTCTTTA ATAGTGGACT CTTGTTCCAA ACTGGAACAA  
CAACCTCAGG TGCAAGAAAT TATCACCTGA GAACAAGGTT TGACCTTGTT

3051 CACTCAACCC TATCTCGGTC TATTCTTTTG ATTTATAAGG GATTTTGCCG  
GTGAGTTGGG ATAGAGCCAG ATAAGAAAAC TAAATATTCC CTAAAACGGC

3101 ATTTTCGGCCT ATTGGTTAAA AAATGAGCTG ATTTAACAAA AATTTAACGC  
TAAAGCCGGA TAACCAATT TTTACTCGAC TAAATTGTTT TTAAATTGCG

3151 GAATTTTAAC AAAATATTAA CGTTTACAAT TTAAATATTT GCTTATACAA  
CTTAAAATTG TTTTATAATT GCAAATGTTA AATTTATAAA CGAATATGTT

3201 TCTTCCTGTT TTTGGGGCTT TTCTGATTAT CAACCGGGGT ACATATGATT  
AGAAGGACAA AAACCCCGAA AAGACTAATA GTTGGCCCCA TGTATACTAA

ClaI

~~~~~

3251 GACATGCTAG TTTTACGATT ACCGTTTCATC GATTCTCTTG TTTGCTCCAG  
CTGTACGATC AAAATGCTAA TGGCAAGTAG CTAAGAGAAC AAACGAGGTC

29/39

3301	ACTCTCAGGC	AATGACCTGA	TAGCCTTTTT	AGACCTCTCA	AAAATAGCTA
	TGAGAGTCCG	TTACTGGACT	ATCGGAAAAA	TCTGGAGAGT	TTTTATCGAT
3351	CCCTCTCCGG	CATGAATTTA	TCAGCTAGAA	CGGTTGAATA	TCATATTGAT
	GGGAGAGGCC	GTACTTAAAT	AGTCGATCTT	GCCAACTTAT	AGTATAACTA
3401	GGTGATTTGA	CTGTCTCCGG	CCTTTCTCAC	CCGTTTGAAT	CTTTACCTAC
	CCACTAAACT	GACAGAGGCC	GGAAAGAGTG	GGCAAACCTA	GAAATGGATG
3451	ACATTACTCA	GGCATTGCAT	TTAAAATATA	TGAGGGTTCT	AAAAATTTTT
	TGTAATGAGT	CCGTAACGTA	AATTTTATAT	ACTCCCAAGA	TTTTTAAAAA
3501	ATCCTTGCGT	TGAAATAAAG	GCTTCTCCCG	CAAAAGTATT	ACAGGGTCAT
	TAGGAACGCA	ACTTTATTTT	CGAAGAGGGC	GTTTTCATAA	TGTCCCAGTA
3551	AATGTTTTTG	GTACAACCGA	TTTAGCTTTA	TGCTCTGAGG	CTTTATTGCT
	TTACAAAAC	CATGTTGGCT	AAATCGAAAT	ACGAGACTCC	GAAATAACGA
3601	TAATTTTGCT	AATTCTTTGC	CTTGCTGTGA	TGATTTATTG	GATGTTAACG
	ATTAAAACGA	TTAAGAAACG	GAACGGACAT	ACTAAATAAC	CTACAATTGC
3651	CTACTACTAT	TAGTAGAATT	GATGCCACCT	TTTCAGCTCG	CGCCCCAAAT
	GATGATGATA	ATCATCTTAA	CTACGGTGGA	AAAGTCGAGC	GCGGGGTTTA
3701	GAAAATATAG	CTAAACAGGT	TATTGACCAT	TTGCGAAATG	TATCTAATGG
	CTTTTATATC	GATTTGTCCA	ATAACTGGTA	AACGCTTTAC	ATAGATTACC
3751	TCAAACATAA	TCTACTCGTT	CGCAGAATTG	GGAATCAACT	GTTACATGGA
	AGTTTGATTT	AGATGAGCAA	GCGTCTTAAC	CCTTAGTTGA	CAATGTACCT
3801	ATGAAACTTC	CAGACACCGT	ACTTTAGTTG	CATATTTAAA	ACATGTTGAG
	TACTTTGAAG	GTCTGTGGCA	TGAAATCAAC	GTATAAATTT	TGTACAACCT
3851	CTACAGCACC	AGATCCAGCA	ATTAAGCTCT	AAGCCATCCG	CAAAAATGAC
	GATGTCGTGG	TCTAGGTCGT	TAATTCGAGA	TTCGGTAGGC	GTTTTTACTG
3901	CTCTTATCAA	AAGGAGCAAT	TAAAGGTACT	CTCTAATCCT	GACCTGTTGG
	GAGAATAGTT	TTCCTCGTTA	ATTTCCATGA	GAGATTAGGA	CTGGACAACC
3951	AGTTTGCTTC	CGGTCTGGTT	CGCTTTGAAG	CTCGAATTAA	AACGCGATAT
	TCAAACGAAG	GCCAGACCAA	GCGAAACTTC	GAGCTTAATT	TTGCGCTATA
4001	TTGAAGTCTT	TCGGGCTTCC	TCTTAATCTT	TTTGATGCAA	TCCGCTTTGC
	AACTTCAGAA	AGCCCGAAGG	AGAATTAGAA	AAACTACGTT	AGGCGAAACG
4051	TTCTGACTAT	AATAGTCAGG	GTAAAGACCT	GATTTTTTGAT	TTATGGTCAT
	AAGACTGATA	TTATCAGTCC	CATTTCTGGA	CTAAAAACTA	AATACCAGTA
4101	TCTCGTTTTT	TGAACTGTTT	AAAGCATTTG	AGGGGGATTC	AATGAATATT
	AGAGCAAAAG	ACTTGACAAA	TTTCGTAAAC	TCCCCCTAAG	TTACTTATAA

30/39

4151	TATGACGATT	CCGCAGTATT	GGACGCTATC	CAGTCTAAAC	ATTTTACTAT
	ATACTGCTAA	GGCGTCATAA	CCTGCGATAG	GTCAGATTTC	TAAAATGATA
4201	TACCCCCTCT	GGCAAAACTT	CTTTTGCAAA	AGCCTCTCGC	TATTTTTGTT
	ATGGGGGAGA	CCGTTTTGAA	GAAAACGTTT	TCGGAGAGCG	ATAAAAACAA
4251	TTTATCGTCG	TCTGGTAAAC	GAGGGTTATG	ATAGTGTTGC	TCTTACTATG
	AAATAGCAGC	AGACCATTTC	CTCCCAATAC	TATCACAACG	AGAATGATAC
4301	CCTCGTAATT	CCTTTTGGCG	TTATGTATCT	GCATTAGTTG	AATGTGGTAT
	GGAGCATTAA	GGAAAACCGC	AATACATAGA	CGTAATCAAC	TTACACCATA
4351	TCCTAAATCT	CAACTGATGA	ATCTTTCTAC	CTGTAATAAT	GTTGTTCCGT
	AGGATTTAGA	GTTGACTACT	TAGAAAGATG	GACATTATTA	CAACAAGGCA
4401	TAGTTCGTTT	TATTAACGTA	GATTTTTCTT	CCCAACGTCC	TGACTGGTAT
	ATCAAGCAAA	ATAATTGCAT	CTAAAAAGAA	GGGTTGCAGG	ACTGACCATA
4451	AATGAGCCAG	TTCTTAAAAT	CGCATAAGGT	AATTCACAAT	GATTAAAGTT
	TTACTCGGTC	AAGAATTTTA	GCGTATTCCA	TTAAGTGTTA	CTAATTTCAA
4501	GAAATTAAAC	CATCTCAAGC	GCAATTCACT	ACCCGTTCTG	GTGTTTCTCG
	CTTTAATTTG	GTAGAGTTTC	CGTTAAGTGA	TGGGCAAGAC	CACAAAGAGC
4551	TCAGGGCAAG	CCTTATTCAC	TGAATGAGCA	GCTTTGTTAC	GTTGATTTGG
	AGTCCCGTTC	GGAATAAGTG	ACTTACTCGT	CGAAACAATG	CAACTAAACC
4601	GTAATGAATA	TCCGGTGCTT	GTCAAGATTA	CTCTTGATGA	AGGTCAGCCA
	CATTACTTAT	AGGCCACGAA	CAGTTCTAAT	GAGAACTACT	TCCAGTCGGT
4651	GCCTATGCGC	CTGGTCTGTA	CACCGTG CAT	CTGTCCTCGT	TCAAAGTTGG
	CGGATACGCG	GACCAGACAT	GTGGCACGTA	GACAGGAGCA	AGTTTCAACC
4701	TCAGTTCGGT	TCTCTTATGA	TTGACCGTCT	GCGCCTCGTT	CCGGCTAAGT
	AGTCAAGCCA	AGAGAATACT	AACTGGCAGA	CGCGGAGCAA	GGCCGATTCA
4751	AACATGGAGC	AGGTCGCGGA	TTTCGACACA	ATTTATCAGG	CGATGATACA
	TTGTACCTCG	TCCAGCGCCT	AAAGCTGTGT	TAAATAGTCC	GCTACTATGT
4801	AATCTCCGTT	GTACTTTGTT	TCGCGCTTGG	TATAATCGCT	GGGGGTCAAA
	TTAGAGGCAA	CATGAAACAA	AGCGCGAACC	ATATTAGCGA	CCCCAGTTT
4851	GATGAGTGTT	TTAGTGTATT	CTTTCGCCTC	TTTCGTTTTA	GGTTGGTGCC
	CTACTCACAA	AATCACATAA	GAAAGCGGAG	AAAGCAAAAT	CCAACCACGG
4901	TTCGTAGTGG	CATTACGTAT	TTTACCCGTT	TAATGGAAAC	TTCCTCATGC
	AAGCATCACC	GTAATGCATA	AAATGGGCAA	ATTACCTTTG	AAGGAGTACG
4951	GTAAGTCTTT	AGTCCTCAAA	GCCTCCGTAG	CCGTTGCTAC	CCTCGTTCGG
	CATTTCAGAAA	TCAGGAGTTT	CGGAGGCATC	GGCAACGATG	GGAGCAAGGC

31/39

5001	ATGCTGTCTT	TCGCTGCTGA	GGGTGACGAT	CCCGCAAAAG	CGGCCTTTGA
	TACGACAGAA	AGCGACGACT	CCCACTGCTA	GGGCGTTTTT	GCCGGAAACT
5051	CTCCCTGCAA	GCCTCAGCGA	CCGAATATAT	CGGTTATGCG	TGGGCGATGG
	GAGGGACGTT	CGGAGTCGCT	GGCTTATATA	GCCAATACGC	ACCCGCTACC
5101	TTGTTGTCAT	TGTCGGCGCA	ACTATCGGTA	TCAAGCTGTT	TAAGAAATTC
	AACAACAGTA	ACAGCCGCGT	TGATAGCCAT	AGTTCGACAA	ATTCTTTAAG
5151	ACCTCGAAAG	CAAGCTGATA	AAGGAGGTTT	CTCGATCGAG	ACGTTGGGTG
	TGGAGCTTTC	GTTCGACTAT	TTCCTCCAAA	GAGCTAGCTC	TGCAACCCAC
5201	AGGTTCCAAC	TTTCACCATA	ATGAAATAAG	ATCACTACCG	GGCGTATTTT
	TCCAAGGTG	AAAGTGGTAT	TACTTTATTC	TAGTGATGGC	CCGCATAAAA
5251	TTGAGTTATC	GAGATTTTCA	GGAGCTAAGG	AAGCTAAAAT	GGAGAAAAAA
	AACTCAATAG	CTCTAAAAGT	CCTCGATTCC	TTGATTTTAA	CCTCTTTTTT
5301	ATCACTGGAT	ATACCACCGT	TGATATATCC	CAATGGCATC	GTAAAGAACA
	TAGTGACCTA	TATGGTGGCA	ACTATATAGG	GTTACCGTAG	CATTTCTTGT
5351	TTTTGAGGCA	TTTCAGTCAG	TTGCTCAATG	TACCTATAAC	CAGACCGTTC
	AAAACCTCCG	AAAGTCAGTC	AACGAGTTAC	ATGGATATTG	GTCTGGCAAG
5401	AGCTGGATAT	TACGGCCTTT	TTAAAGACCG	TAAAGAAAAA	TAAGCACAAG
	TCGACCTATA	ATGCCGGAAA	AATTTCTGGC	ATTTCTTTTT	ATTCGTGTTC
5451	TTTTATCCGG	CCTTTATTCA	CATTCTTGCC	CGCCTGATGA	ATGCTCATCC
	AAAATAGGCC	GGAAATAAGT	GTAAGAACGG	GCGGACTACT	TACGAGTAGG
5501	GGAGTTCCGT	ATGGCAATGA	AAGACGGTGA	GCTGGTGATA	TGGGATAGTG
	CCTCAAGGCA	TACCGTTACT	TTCTGCCACT	CGACCACTAT	ACCCTATCAC
5551	TTACACCCTG	TTACACCGTT	TTCCATGAGC	AAACTGAAAC	GTTTTTCATC
	AAGTGGGAAC	AATGTGGCAA	AAGGTACTCG	TTTGACTTTG	CAAAAGTAGC
5601	CTCTGGAGTG	AATACCACGA	CGATTTCCGG	CAGTTTCTAC	ACATATATTC
	GAGACCTCAC	TTATGGTGCT	GCTAAAGGCC	GTCAAAGATG	TGTATATAAG
5651	GCAAGATGTG	GCGTGTTACG	GTGAAAACCT	GGCCTATTTT	CCTAAAGGGT
	CGTTCTACAC	CGCACAAATG	CACTTTTGGG	CCGGATAAAG	GGATTTCCTA
5701	TTATTGAGAA	TATGTTTTTC	GTCTCAGCCA	ATCCCTGGGT	GAGTTTCACC
	AATAACTCTT	ATACAAAAAG	CAGAGTCGGT	TAGGGACCCA	CTCAAAGTGG
5751	AGTTTTGATT	TAAACGTAGC	CAATATGGAC	AACTTCTTCG	CCCCCGTTTT
	TCAAAACTAA	ATTTGCATCG	GTTATACCTG	TTGAAGAAGC	GGGGGCAAAA
5801	CACTATGGGC	AAATATTATA	CGCAAGGCGA	CAAGGTGCTG	ATGCCGCTGG
	GTGATACCCG	TTTATAATAT	GCGTTCCGCT	GTTCCACGAC	TACGGCGACC

32/39

5851	CGATTCAGGT	TCATCATGCC	GTTTGTGATG	GCTTCCATGT	CGGCAGAATG
	GCTAAGTCCA	AGTAGTACGG	CAAACACTAC	CGAAGGTACA	GCCGTCTTAC
5901	CTTAATGAAT	TACAACAGTA	CTGCGATGAG	TGGCAGGGCG	GGGCGTAATT
	GAATTACTTA	ATGTTGTCAT	GACGCTACTC	ACCGTCCCGC	CCCGCATTAA
5951	TTTTTAAGGC	AGTTATTGGT	GCCCTTAAAC	GCCTGGTGCT	AGCCTGAGGC
	AAAAATTCCG	TCAATAACCA	CGGGAATTTG	CGGACCACGA	TCGGACTCCG
6001	CAGTTTGCTC	AGGCTCTCCC	CGTGGAGGTA	ATAATTGCTC	GACCGATAAA
	GTCAAACGAG	TCCGAGAGGG	GCACCTCCAT	TATTAACGAG	CTGGCTATTT
6051	AGCGGCTTCC	TGACAGGAGG	CCGTTTTGTT	TTGCAGCCCA	CCTCAACGCA
	TCGCCGAAGG	ACTGTCTCTC	GGCAAAACAA	AACGTCGGGT	GGAGTTGCGT
6101	ATTAATGTGA	GTTAGCTCAC	TCATTAGGCA	CCCCAGGCTT	TACACTTTAT
	TAATTACACT	CAATCGAGTG	AGTAATCCGT	GGGGTCCGAA	ATGTGAAATA
6151	GCTTCCGGCT	CGTATGTTGT	GTGGAATTGT	GAGCGGATAA	CAATTTACACA
	CGAAGGCCGA	GCATACAACA	CACCTTAACA	CTCGCCTATT	GTTAAAGTGT
6201	CAGGAAACAG	CTATGACCAT	GATTACGAAT	TTCTAGATAA	CGAGGGCAAA
	GTCCTTTGTC	GATACTGGTA	CTAATGCTTA	AAGATCTATT	GCTCCCGTTT
6251	AAATGAAAAA	GACAGCTATC	GCGATTGCAG	TGGCACTGGC	TGGTTTCGCT
	TTTACTTTTT	CTGTTCGATAG	CGCTAACGTC	ACCGTGACCG	ACCAAAGCGA
6301	ACCGTAGCGC	AGGCCGACTA	CAAAGATGTC	GACTGTATTG	TTTATCATGC
	TGGCATCGCG	TCCGGCTGAT	GTTTCTACAG	CTGACATAAC	AAATAGTACG
				BamHI EcoRI	
				~~~~~	
6351	TCATTATCTT	GTTGCTAAGT	GTGGTGGTGG	AGGATCCGAA	TTCAATGCTG
	AGTAATAGAA	CAACGATTCA	CACCACCACC	TCCTAGGCTT	AAGTTACGAC
6401	GCGGCGGCTC	TGGTGGTGGT	TCTGGTGGCG	GCTCTGAGGG	TGGTGGCTCT
	CGCCGCCGAG	ACCACCACCA	AGACCACCGC	CGAGACTCCC	ACCACCGAGA
6451	GAGGGTGGCG	GTTCTGAGGG	TGGCGGCTCT	GAGGGAGGCG	GTTCCGGTGG
	CTCCCACCGC	CAAGACTCCC	ACCGCCGAGA	CTCCCTCCGC	CAAGGCCACC
6501	TGGCTCTGGT	TCCGGTGATT	TTGATTATGA	AAAGATGGCA	AACGCTAATA
	ACCGAGACCA	AGGCCACTAA	AACTAATACT	TTTCTACCGT	TTGCGATTAT
6551	AGGGGGCTAT	GACCGAAAAT	GCCGATGAAA	ACGCGCTACA	GTCTGACGCT
	TCCCCCGATA	CTGGCTTTTA	CGGCTACTTT	TGCGCGATGT	CAGACTGCGA

33/39

ClaI

~~~~~

```
6601  AAAGGCAAAC TTGATTCTGT CGCTACTGAT TACGGTGCTG CTATCGATGG
      TTTCCGTTTG AACTAAGACA GCGATGACTA ATGCCACGAC GATAGCTACC

6651  TTTCATTGGT GACGTTTCCG GCCTTGCTAA TGGTAATGGT GCTACTGGTG
      AAAGTAACCA CTGCAAAGGC CGGAACGATT ACCATTACCA CGATGACCAC

6701  ATTTTGCTGG CTCTAATTCC CAAATGGCTC AAGTCGGTGA CGGTGATAAT
      TAAAACGACC GAGATTAAGG GTTTACCGAG TTCAGCCACT GCCACTATTA

6751  TCACCTTTAA TGAATAATTT CCGTCAATAT TTACCTTCCC TCCCTCAATC
      AGTGGAAATT ACTTATTAAA GGCAGTTATA AATGGAAGGG AGGGAGTTAG

6801  GGTGAATGT  CGCCCTTTTG TCTTTGGCGC TGGTAAACCA TATGAATTTT
      CCAACTTACA GCGGGAAAAC AGAAACCGCG ACCATTTGGT ATACTTAAAA

6851  CTATTGATTG TGACAAAATA AACTTATTCC GTGGTGTCTT TCGGTTTCTT
      GATAACTAAC ACTGTTTTAT TTGAATAAGG CACCACAGAA ACGCAAAGAA

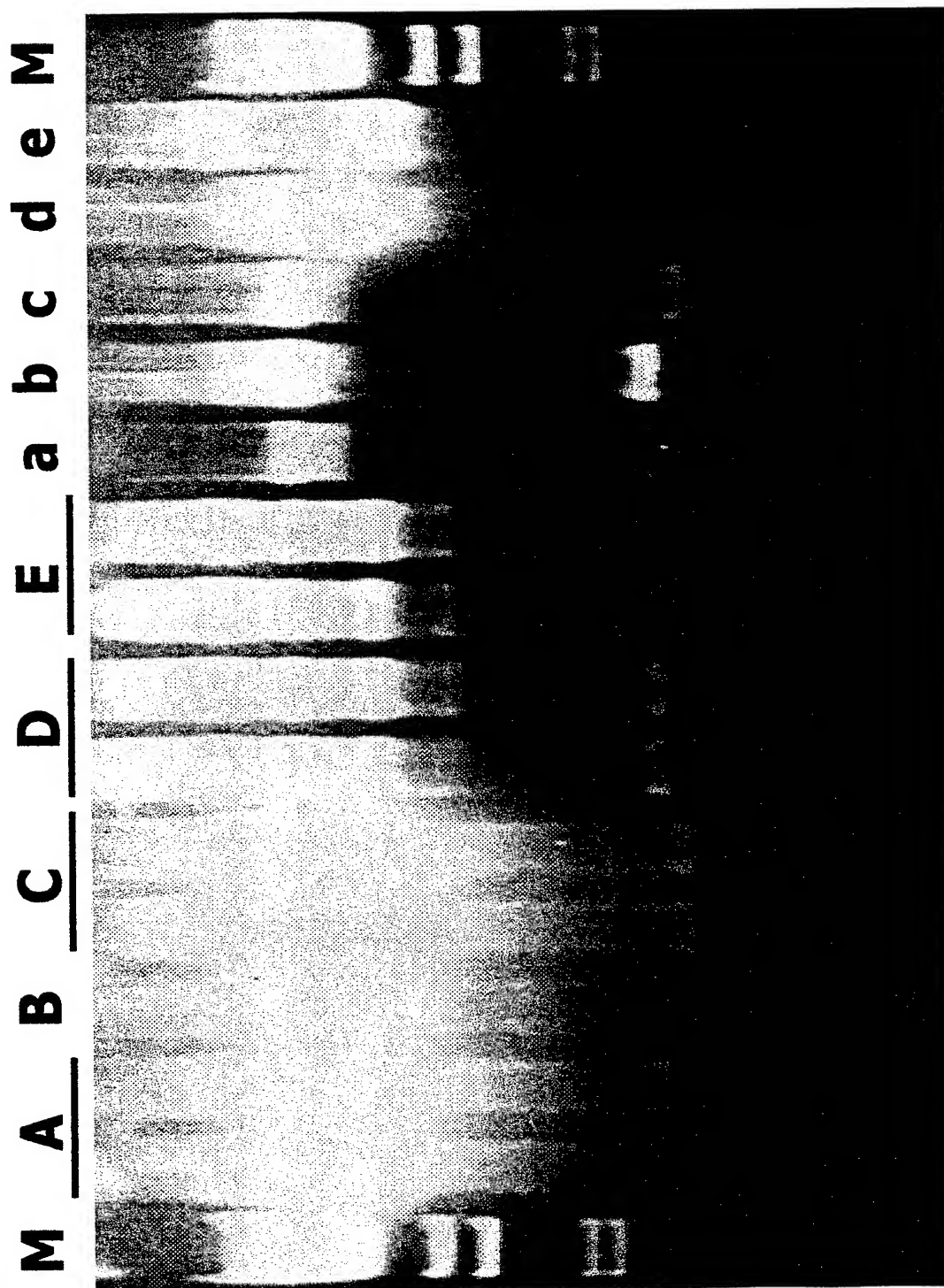
6901  TTATATGTTG CCACCTTTAT GTATGTATTT TCTACGTTTG CTAACATACT
      AATATACAAC GGTGGAAATA CATAcataaa AGATGCAAAC GATTGTATGA
```

HindIII

```
6951  GCGTAATAAG GAGTCTTGAT A
      CGCATTATTC CTCAGAACTA T
```

34/39

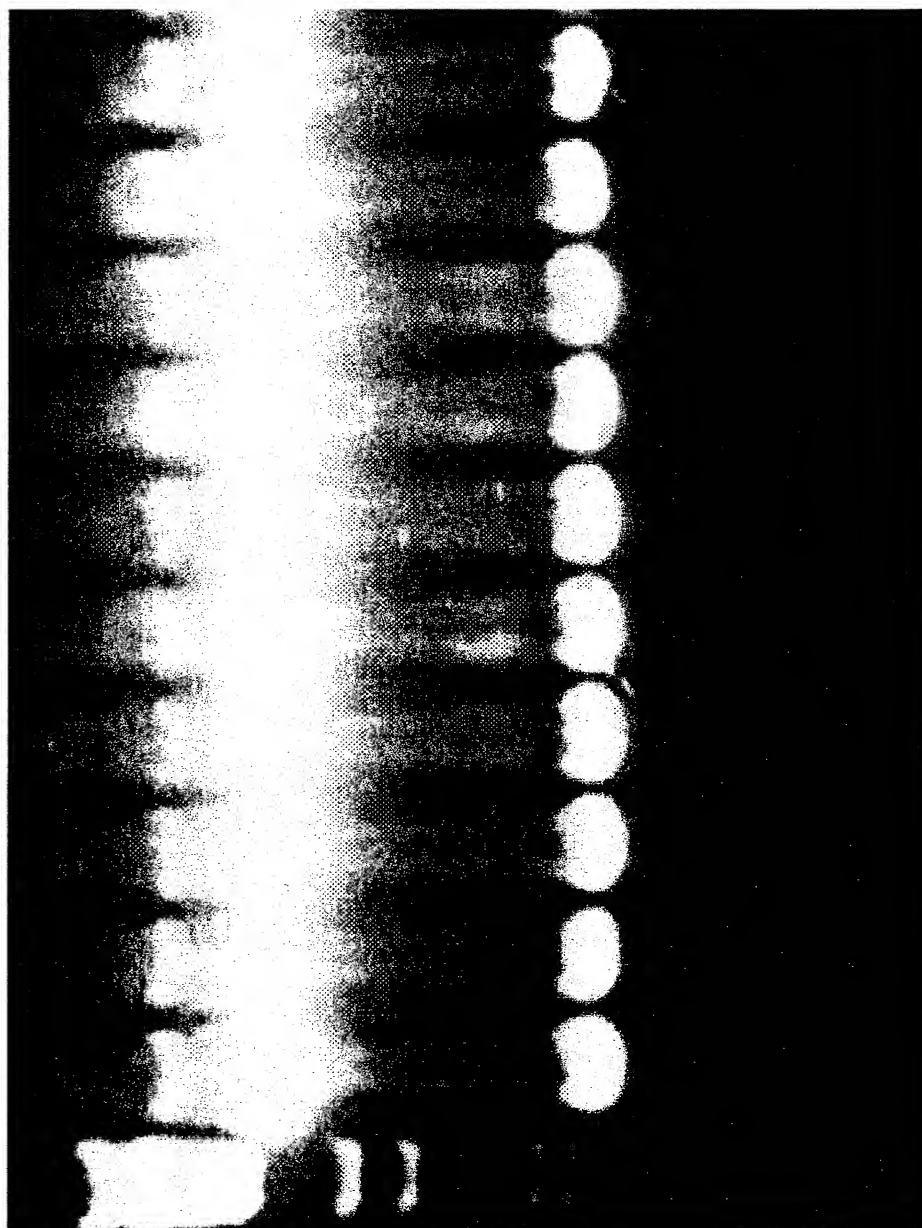
**Figure 5**



35/39

**Figure 6**

**CO-  
M SIP Polypheage transductants transf.**



**1 2 3 4 5 6 7 8 9 10**

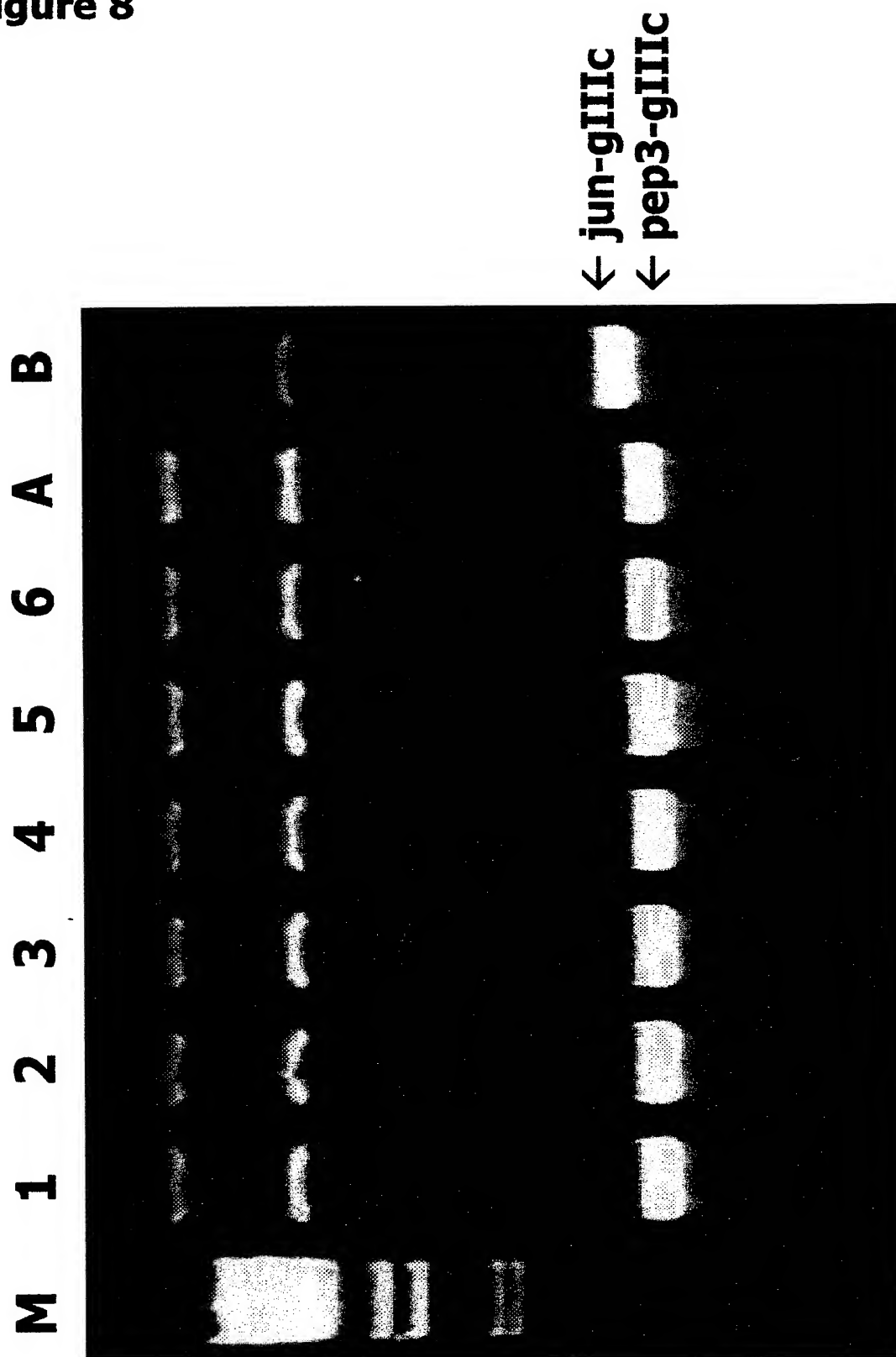


36/39

**Figure 7**

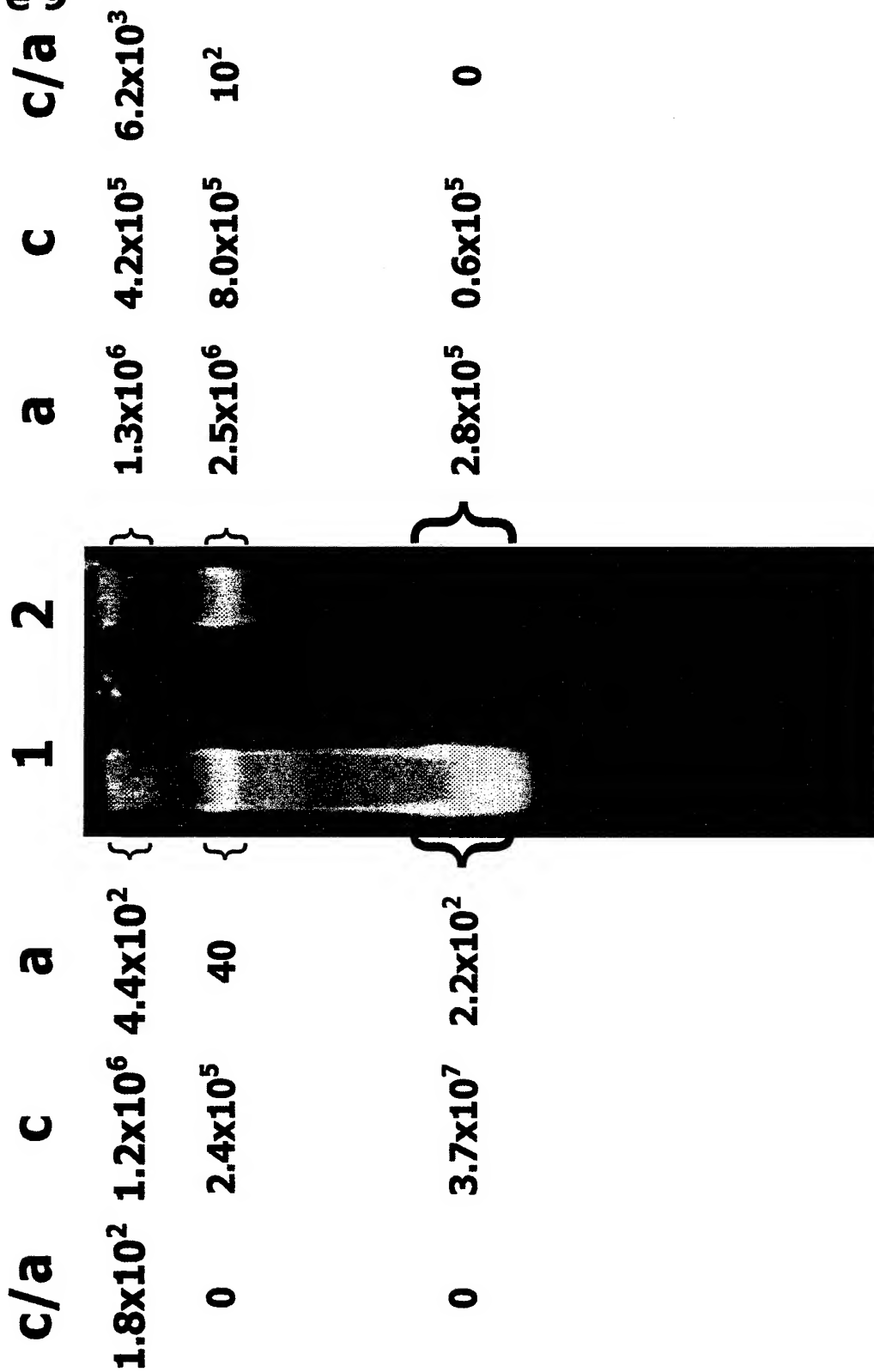
| <b>dilution factor</b> |                       | <b>transductants</b> |                             |
|------------------------|-----------------------|----------------------|-----------------------------|
| <b>pep3/p75ICD</b>     | <b>jun/p75ICD</b>     | <b>(t.u./ml)*</b>    |                             |
| <b>1</b>               | <b>pos. control</b>   | <b>-</b>             | <b>6 x 10<sup>5</sup></b>   |
| <b>-</b>               | <b>neg. control</b>   | <b>1</b>             | <b>0</b>                    |
| <b>1</b>               | <b>10<sup>2</sup></b> |                      | <b>1.2 x 10<sup>4</sup></b> |
| <b>1</b>               | <b>10<sup>3</sup></b> |                      | <b>8.6 x 10<sup>2</sup></b> |
| <b>1</b>               | <b>10<sup>4</sup></b> |                      | <b>1.2 x 10<sup>2</sup></b> |
| <b>1</b>               | <b>10<sup>5</sup></b> |                      | <b>12<sup>#</sup></b>       |
| <b>1</b>               | <b>10<sup>6</sup></b> |                      | <b>1.2<sup>#</sup></b>      |
| <b>1</b>               | <b>10<sup>7</sup></b> |                      | <b>0.12<sup>#</sup></b>     |

37/39

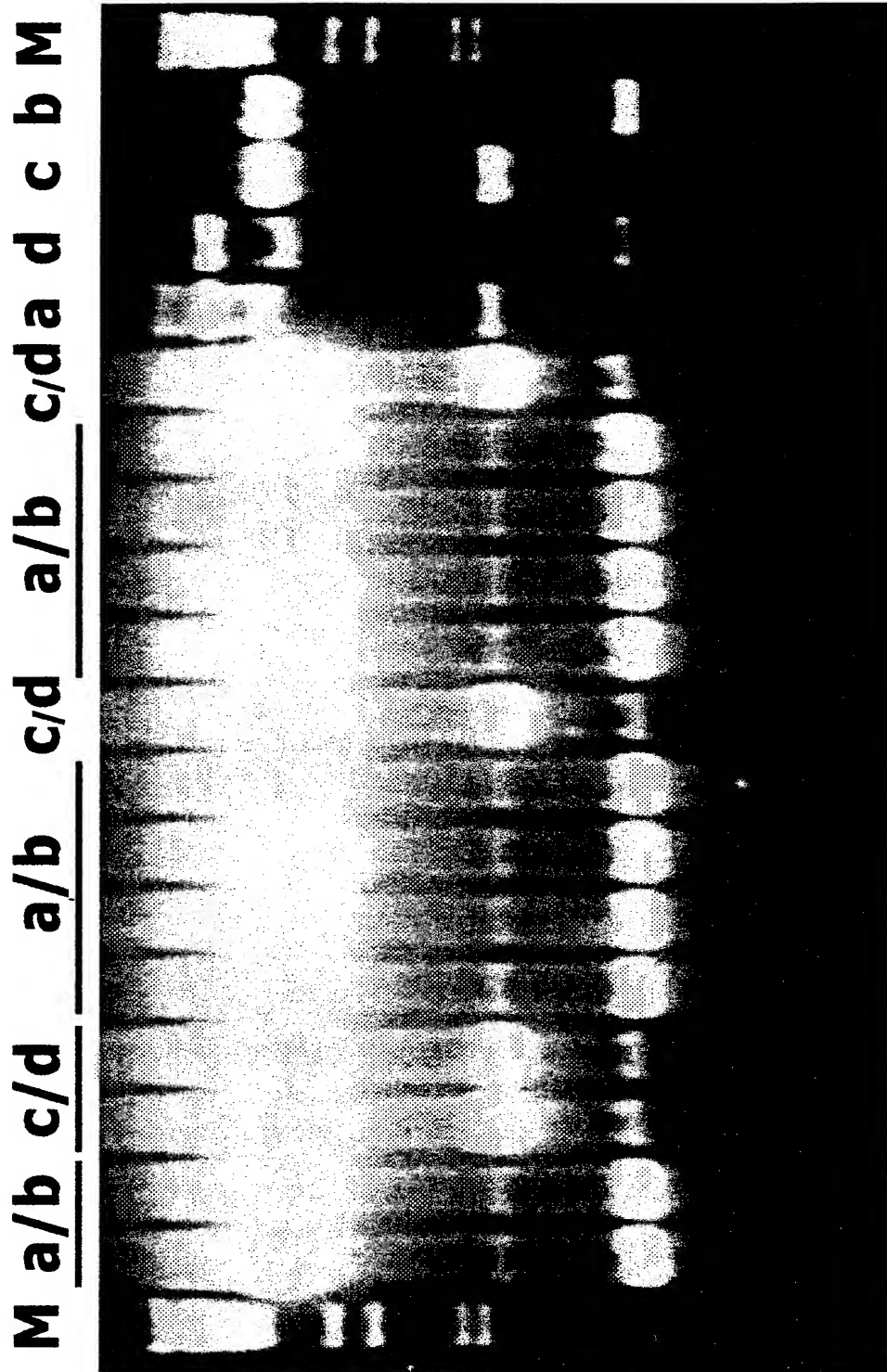
**Figure 8**

38/39

Figure 9



39/39

**Figure 10**



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                         |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------|
| <b>(51) International Patent Classification <sup>6</sup> :</b><br><b>A61K 38/00, 38/28, 9/26, 33/06</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | <b>A1</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | <b>(11) International Publication Number:</b> <b>WO 99/27944</b><br><b>(43) International Publication Date:</b> 10 June 1999 (10.06.99) |
| <b>(21) International Application Number:</b> PCT/US98/25386<br><b>(22) International Filing Date:</b> 30 November 1998 (30.11.98)<br><br><b>(30) Priority Data:</b><br>60/067,740                      2 December 1997 (02.12.97)      US<br>60/080,970                      7 April 1998 (07.04.98)          US<br><br><b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Applications</b><br>US                                              60/080,970 (CIP)<br>Filed on                                      7 April 1998 (07.04.98)<br>US                                              60/067,740 (CIP)<br>Filed on                                      2 December 1997 (02.12.97)<br><br><b>(71) Applicant (for all designated States except US):</b> ATHENA NEUROSCIENCES, INC. [US/US]; 800 F. Gateway Boulevard, South San Francisco, CA 94080 (US).<br><br><b>(72) Inventor; and</b><br><b>(75) Inventor/Applicant (for US only):</b> SCHENK, Dale, B. [US/US]; 1542 Los Altos Drive, Burlingame, CA 94010-5941 (US). | <b>(74) Agents:</b> LIEBESCHUETZ, Joe et al.; Townsend and Townsend and Crew LLP, 8th floor, Two Embarcadero Center, San Francisco, CA 94111 (US).<br><br><b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).<br><br><b>Published</b><br><i>With international search report.</i><br><i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> |                                                                                                                                         |
| <b>(54) Title:</b> PREVENTION AND TREATMENT OF AMYLOIDOGENIC DISEASE<br><br><b>(57) Abstract</b><br><p>The invention provides compositions and methods for treatment of amyloidogenic diseases. Such methods entail administering an agent that induces a beneficial immune response against an amyloid deposit in the patient. The methods are particularly useful for prophylactic and therapeutic treatment of Alzheimer's disease. In such methods, a suitable agent is A<math>\beta</math> peptide or an antibody thereto.</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                                                                                                                                         |

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

|    |                          |    |                                          |    |                                              |    |                          |
|----|--------------------------|----|------------------------------------------|----|----------------------------------------------|----|--------------------------|
| AL | Albania                  | ES | Spain                                    | LS | Lesotho                                      | SI | Slovenia                 |
| AM | Armenia                  | FI | Finland                                  | LT | Lithuania                                    | SK | Slovakia                 |
| AT | Austria                  | FR | France                                   | LU | Luxembourg                                   | SN | Senegal                  |
| AU | Australia                | GA | Gabon                                    | LV | Latvia                                       | SZ | Swaziland                |
| AZ | Azerbaijan               | GB | United Kingdom                           | MC | Monaco                                       | TD | Chad                     |
| BA | Bosnia and Herzegovina   | GE | Georgia                                  | MD | Republic of Moldova                          | TG | Togo                     |
| BB | Barbados                 | GH | Ghana                                    | MG | Madagascar                                   | TJ | Tajikistan               |
| BE | Belgium                  | GN | Guinea                                   | MK | The former Yugoslav<br>Republic of Macedonia | TM | Turkmenistan             |
| BF | Burkina Faso             | GR | Greece                                   | ML | Mali                                         | TR | Turkey                   |
| BG | Bulgaria                 | HU | Hungary                                  | MN | Mongolia                                     | TT | Trinidad and Tobago      |
| BJ | Benin                    | IE | Ireland                                  | MR | Mauritania                                   | UA | Ukraine                  |
| BR | Brazil                   | IL | Israel                                   | MW | Malawi                                       | UG | Uganda                   |
| BY | Belarus                  | IS | Iceland                                  | MX | Mexico                                       | US | United States of America |
| CA | Canada                   | IT | Italy                                    | NE | Niger                                        | UZ | Uzbekistan               |
| CF | Central African Republic | JP | Japan                                    | NL | Netherlands                                  | VN | Viet Nam                 |
| CG | Congo                    | KE | Kenya                                    | NO | Norway                                       | YU | Yugoslavia               |
| CH | Switzerland              | KG | Kyrgyzstan                               | NZ | New Zealand                                  | ZW | Zimbabwe                 |
| CI | Côte d'Ivoire            | KP | Democratic People's<br>Republic of Korea | PL | Poland                                       |    |                          |
| CM | Cameroon                 | KR | Republic of Korea                        | PT | Portugal                                     |    |                          |
| CN | China                    | KZ | Kazakistan                               | RO | Romania                                      |    |                          |
| CU | Cuba                     | LC | Saint Lucia                              | RU | Russian Federation                           |    |                          |
| CZ | Czech Republic           | LI | Liechtenstein                            | SD | Sudan                                        |    |                          |
| DE | Germany                  | LK | Sri Lanka                                | SE | Sweden                                       |    |                          |
| DK | Denmark                  | LR | Liberia                                  | SG | Singapore                                    |    |                          |
| EE | Estonia                  |    |                                          |    |                                              |    |                          |

## PREVENTION AND TREATMENT OF AMYLOIDOGENIC DISEASE

### CROSS-REFERENCE TO RELATED APPLICATIONS

This application derives priority from USSN 60/067,740, filed December 2, 1997, and USSN 60/080,970, filed April 7, 1998, which are incorporated by reference in their entirety for all purposes.

5

### TECHNICAL FIELD

The invention resides in the technical fields of immunology and medicine.

### BACKGROUND

10 Alzheimer's disease (AD) is a progressive disease resulting in senile dementia. See generally Selkoe, *TINS* 16, 403-409 (1993); Hardy et al., WO 92/13069; Selkoe, *J. Neuropathol. Exp. Neurol.* 53, 438-447 (1994); Duff et al., *Nature* 373, 476-477 (1995); Games et al., *Nature* 373, 523 (1995). Broadly speaking the disease falls into two categories: late onset, 15 which occurs in old age (65 + years) and early onset, which develops well before the senile period, i.e, between 35 and 60 years. In both types of disease, the pathology is the same but the abnormalities tend to be more severe and widespread in cases beginning at an earlier age. The disease is 20 characterized by two types of lesions in the brain, senile plaques and neurofibrillary tangles. Senile plaques are areas of disorganized neuropil up to 150  $\mu$ m across with extracellular amyloid deposits at the center visible by microscopic analysis of sections of brain tissue. 25 Neurofibrillary tangles are intracellular deposits of tau protein consisting of two filaments twisted about each other in pairs.

The principal constituent of the plaques is a peptide termed A $\beta$  or  $\beta$ -amyloid peptide. A $\beta$  peptide is an internal fragment of 39-43 amino acids of a precursor protein termed amyloid precursor protein (APP). Several mutations within the APP protein have been correlated with the presence of Alzheimer's disease. See, e.g., Goate et al., *Nature* 349, 704 (1991) (valine<sup>717</sup> to isoleucine); Chartier Harlan et al. *Nature* 353, 844 (1991) (valine<sup>717</sup> to glycine); Murrell et al., *Science* 254, 97 (1991) (valine<sup>717</sup> to phenylalanine); Mullan et al., *Nature Genet.* 1, 345 (1992) (a double mutation changing lysine<sup>595</sup>-methionine<sup>596</sup> to asparagine<sup>595</sup>-leucine<sup>596</sup>). Such mutations are thought to cause Alzheimer's disease by increased or altered processing of APP to A $\beta$ , particularly processing of APP to increased amounts of the long form of A $\beta$  (i.e., A $\beta$ 1-42 and A $\beta$ 1-43). Mutations in other genes, such as the presenilin genes, PS1 and PS2, are thought indirectly to affect processing of APP to generate increased amounts of long form A $\beta$  (see Hardy, *TINS* 20, 154 (1997)). These observations indicate that A $\beta$ , and particularly its long form, is a causative element in Alzheimer's disease.

McMichael, EP 526,511, proposes administration of homeopathic dosages (less than or equal to  $10^{-2}$  mg/day) of A $\beta$  to patients with preestablished AD. In a typical human with about 5 liters of plasma, even the upper limit of this dosage would be expected to generate a concentration of no more than 2 pg/ml. The normal concentration of A $\beta$  in human plasma is typically in the range of 50-200 pg/ml (Seubert et al., *Nature* 359, 325-327 (1992)). Because EP 526,511's proposed dosage would barely alter the level of endogenous circulating A $\beta$  and because EP 526,511 does not recommend use of an adjuvant, it seems implausible that any therapeutic benefit would result.

By contrast, the present invention is directed *inter alia* to treatment of Alzheimer's and other amyloidogenic diseases by administration of A $\beta$  or other immunogen to a patient under conditions that generate a beneficial immune response in the patient. The invention thus fulfills a longstanding need for therapeutic regimes for preventing or ameliorating the neuropathology of Alzheimer's disease.



## SUMMARY OF THE CLAIMED INVENTION

In one aspect, the invention provides methods of preventing or treating a disease characterized by amyloid deposition in a patient. Such methods entail inducing an immune response  
5 against a peptide component of an amyloid deposit in the patient. Such induction can be active by administration of an immunogen or passive by administration of an antibody or an active fragment or derivative of the antibody. In some patients, the amyloid deposit is aggregated A $\beta$  peptide and  
10 the disease is Alzheimer's disease. In some methods, the patient is asymptomatic. In some methods, the patient is under 50 years of age. In some methods, the patient has inherited risk factors indicating susceptibility to Alzheimer's disease. Such risk factors include variant  
15 alleles in presenilin gene PS1 or PS2 and variant forms of APP. In other methods, the patient has no known risk factors for Alzheimer's disease.

For treatment of patients suffering from Alzheimer's disease, one treatment regime entails administering a dose of  
20 A $\beta$  peptide to the patient to induce the immune response. In some methods, the A $\beta$  peptide is administered with an adjuvant that enhances the immune response to the A $\beta$  peptide. In some methods, the adjuvant is alum. In some methods, the adjuvant is MPL. The dose of A $\beta$  peptide administered to the patient is  
25 typically at least 1 or 10  $\mu$ g, if administered with adjuvant, and at least 50  $\mu$ g if administered without adjuvant. In some methods, the dose is at least 100  $\mu$ g.

In some methods, the A $\beta$  peptide is A $\beta$ 1-42. In some methods, the A $\beta$  peptide is administered in aggregated form.  
30 In other methods, the A $\beta$  peptide is administered in dissociated form. In some methods, the therapeutic agent is an effective dose of a nucleic acid encoding A $\beta$  or an active fragment or derivative thereof. The nucleic acid encoding A $\beta$  or fragment thereof is expressed in the patient to produce A $\beta$   
35 or the active fragment thereof, which induces the immune response. In some such methods, the nucleic acid is administered through the skin, optionally via a patch. In some methods, a therapeutic agent is identified by screening a

library of compounds to identify a compound reactive with antibodies to A $\beta$ , and administering the compound to the patient to induce the immune response.

In some methods, the immune response is directed to aggregated A $\beta$  peptide without being directed to dissociated A $\beta$  peptide. For example, the immune response can comprise antibodies that bind to aggregated A $\beta$  peptide without binding to dissociated A $\beta$  peptide. In some methods, the immune response comprises T-cells that bind to A $\beta$  complexed with MCH1 or MHCII on CD8 or CD4 cells. In other methods, the immune response is induced by administering an antibody to A $\beta$  to the patient. In some methods, the immune response is induced by removing T-cells from the patient, contacting the T-cells with A $\beta$  peptide under conditions in which the T-cells are primed, and replacing the T-cells in the patient.

The therapeutic agent is typically administered orally, intranasally, intradermally, subcutaneously, intramuscularly, topically or intravenously. In some methods, the patient is monitored followed administration to assess the immune response. If the monitoring indicates a reduction of the immune response over time, the patient can be given one or more further doses of the agent.

In another aspect, the invention provides pharmaceutical compositions comprising A $\beta$  and an excipient suitable for oral and other routes of administration. The invention also provides pharmaceutical compositions comprising an agent effective to induce an immunogenic response against A $\beta$  in a patient, and a pharmaceutically acceptable adjuvant. In some such compositions, the agent is A $\beta$  or an active fragment thereof. In some compositions, the adjuvant comprises alum. In some compositions, the adjuvant comprises an oil-in-water emulsion. In some compositions, the A $\beta$  or active fragment is a component of a polylactide polyglycolide copolymer (PLPG) or other particle. The invention further provides compositions comprising A $\beta$  or an active fragment linked to a conjugate molecule that promotes delivery of A $\beta$  to the bloodstream of a patient and/or promotes an immune response against A $\beta$ . For example, the conjugate can serve to promote an immune response

against A $\beta$ . In some compositions, the conjugate is cholera toxin. In some compositions, the conjugate is an immunoglobulin. In some compositions, the conjugate is attenuated diphtheria toxin CRM 197 (Gupta, Vaccine 15, 1341-3 (1997)).

The invention also provides pharmaceutical compositions comprising an agent effect to induce an immunogenic response against A $\beta$  in a patient with the proviso that the composition is free of Complete Freund's adjuvant. The invention also provides compositions comprising a viral vector encoding A $\beta$  or a an active fragment thereof effective to induce an immune response against A $\beta$ . Suitable viral vectors include herpes, adenovirus, adenoassociated virus, a retrovirus, sindbis, semiliki forest virus, vaccinia or avian pox.

The invention further provides methods of preventing or treating Alzheimer's disease. In such methods, an effective dose of A $\beta$  peptide is administered to a patient. The invention further provides for the use of A $\beta$ , or an antibody thereto, in the manufacture of a medicament for prevention or treatment of Alzheimer's disease.

In another aspect, the invention provides methods of assessing efficacy of an Alzheimer's treatment method in a patient. In these methods, a baseline amount of antibody specific for A $\beta$  peptide is determined in a tissue sample from the patient before treatment with an agent. An amount of antibody specific for A $\beta$  peptide in the tissue sample from the patient after treatment with the agent is compared to the baseline amount of A $\beta$  peptide-specific antibody. An amount of A $\beta$  peptide-specific antibody measured after the treatment that is significantly greater than the baseline amount of A $\beta$  peptide-specific antibody indicates a positive treatment outcome.

In others methods of assessing efficacy of an Alzheimer's treatment method in a patient, a baseline amount of antibody specific for A $\beta$  peptide in a tissue sample from a patient before treatment with an agent is determined. An amount of antibody specific for A $\beta$  peptide in the tissue sample from the subject after treatment with the agent is compared to the

baseline amount of A $\beta$  peptide-specific antibody. A reduction or lack of significant difference between the amount of A $\beta$  peptide-specific antibody measured after the treatment compared to the baseline amount of A $\beta$  peptide-specific antibody indicates a negative treatment outcome.

In other methods of assessing efficacy of an Alzheimer's disease treatment method in a patient a control amount of antibody specific for A $\beta$  peptide is determined in tissue samples from a control population. An amount of antibody specific for A $\beta$  peptide in a tissue sample from the patient after administering an agent is compared to the control amount of A $\beta$  peptide-specific antibody. An amount of A $\beta$  peptide-specific antibody measured after the treatment that is significantly greater than the control amount of A $\beta$  peptide-specific antibody indicates a positive treatment outcome.

In other methods of assessing efficacy of an Alzheimer's treatment method in a patient, a control amount of antibody specific for A $\beta$  peptide in tissues samples from a control population is determined. An amount of antibody specific for A $\beta$  peptide in a tissue sample from the patient after administering an agent is compared to the control amount of A $\beta$  peptide-specific antibody. A lack of significant difference between the amount of A $\beta$  peptide-specific antibody measured after beginning said treatment compared to the control amount of A $\beta$  peptide-specific antibody indicates a negative treatment outcome.

Other methods of monitoring Alzheimer's disease or susceptibility thereto in a patient, comprise detecting an immune response against A $\beta$  peptide in a sample from the patient. In some such methods, the patient is being administered an agent effective to treat or prevent Alzheimer's disease, and the level of the response determines the future treatment regime of the patient.

In other methods of assessing efficacy of an Alzheimer's treatment method in a patient a value for an amount of antibody specific for A $\beta$  peptide in tissue sample from a patient who has been treated with an agent is determined. The

value is compared with a control value determined from a population of patient experiencing amelioration of, or freedom from, symptoms of Alzheimer's disease due to treatment with the agent. A value in the patient at least equal to the control value indicates a positive response to treatment.

The invention further provides diagnostic kits for performing the above methods. Such kits typically include a reagent that specifically binds to antibodies to A $\beta$  or which stimulates proliferation of T-cells reactive with A $\beta$ .

#### BRIEF DESCRIPTION OF THE FIGURES

Fig. 1: Antibody titer after injection of transgenic mice with A $\beta$ 1-42.

Fig. 2: Amyloid burden in the hippocampus. The percentage of the area of the hippocampal region occupied by amyloid plaques, defined by reactivity with the A $\beta$ -specific mA $\beta$  3D6, was determined by computer-assisted quantitative image analysis of immunoreacted brain sections. The values for individual mice are shown sorted by treatment group. The horizontal line for each grouping indicates the median value of the distribution.

Fig 3: Neuritic dystrophy in the hippocampus. The percentage of the area of the hippocampal region occupied by dystrophic neurites, defined by their reactivity with the human APP-specific mA $\beta$  8E5, was determined by quantitative computer-assisted image analysis of immunoreacted brain sections. The values for individual mice are shown for the AN1792-treated group and the PBS-treated control group. The horizontal line for each grouping indicates the median value of the distribution.

Fig. 4: Astrocytosis in the retrosplenial cortex. The percentage of the area of the cortical region occupied by glial fibrillary acidic protein (GFAP)-positive astrocytes was determined by quantitative computer-assisted image analysis of immunoreacted brain sections. The values for individual mice are shown sorted by treatment group and median group values are indicated by horizontal lines.

Fig. 5: Geometric mean antibody titers to A $\beta$ 1-42 following immunization with a range of eight doses of AN1792 containing 0.14, 0.4, 1.2, 3.7, 11, 33, 100, or 300  $\mu$ g.

Fig. 6: Kinetics of antibody response to AN1792 immunization. Titers are expressed as geometric means of values for the 6 animals in each group.

Fig. 7: Quantitative image analysis of the cortical amyloid burden in PBS- and AN1792-treated mice.

Fig. 8: Quantitative image analysis of the neuritic plaque burden in PBS- and AN1792-treated mice.

Fig. 9: Quantitative image analysis of the percent of the retrosplenial cortex occupied by astrogliosis in PBS- and AN1792-treated mice.

Fig. 10: Lymphocyte Proliferation Assay on spleen cells from AN1792-treated (upper panel) or PBS-treated (lower panel).

Fig. 11: Total A $\beta$  levels in the cortex. A scatterplot of individual A $\beta$  profiles in mice immunized with A $\beta$  or APP derivatives combined with Freund's adjuvant.

Fig. 12: Amyloid burden in the cortex was determined by quantitative image analysis of immunoreacted brain sections for mice immunized with the A $\beta$  peptide conjugates A $\beta$ 1-5, A $\beta$ 1-12, and A $\beta$ 13-28; the full length A $\beta$  aggregates AN1792 (A $\beta$ 1-42) and AN1528 (A $\beta$ 1-40) and the PBS-treated control group.

Fig. 13: Geometric mean titers of A $\beta$ -specific antibody for groups of mice immunized with A $\beta$  or APP derivatives combined with Freund's adjuvant.

Fig. 14: Geometric mean titers of A $\beta$ -specific antibody for groups of guinea pigs immunized with AN1792, or a palmitoylated derivative thereof, combined with various adjuvants.

Fig. 15: A $\beta$  levels in the cortex of 12-month old PDAPP mice treated with AN1792 or AN1528 with different adjuvants.

#### DEFINITIONS

The term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 65

percent sequence identity, preferably at least 80 or 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity or higher). Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (see generally Ausubel et al., *supra*). One example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., *J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). Typically, default program parameters can be used to perform the sequence comparison, although customized parameters can also be used. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89, 10915 (1989)).

For purposes of classifying amino acids substitutions as conservative or nonconservative, amino acids are grouped as follows: Group I (hydrophobic sidechains): norleucine, met, ala, val, leu, ile; Group II (neutral hydrophilic side chains): cys, ser, thr; Group III (acidic side chains): asp, glu; Group IV (basic side chains): asn, gln, his, lys, arg; Group V (residues influencing chain orientation): gly, pro; and Group VI (aromatic side chains): trp, tyr, phe. Conservative substitutions involve substitutions between amino acids in the same class. Non-conservative substitutions constitute exchanging a member of one of these classes for a member of another.

Therapeutic agents of the invention are typically substantially pure. This means that an agent is typically at least about 50% w/w (weight/weight) purity, as well as being substantially free from interfering proteins and contaminants. Sometimes the agents are at least about 80% w/w and, more preferably at least 90 or about 95% w/w purity. However, using conventional protein purification techniques, homogeneous peptides of at least 99% w/w can be obtained.

Specific binding between two entities means an affinity of at least  $10^6$ ,  $10^7$ ,  $10^8$ ,  $10^9$  M<sup>-1</sup>, or  $10^{10}$  M<sup>-1</sup>. Affinities greater than  $10^8$  M<sup>-1</sup> are preferred.

The term "antibody" is used to include intact antibodies and binding fragments thereof. Typically, fragments compete with the intact antibody from which they were derived for specific binding to an antigen. Optionally, antibodies or binding fragments thereof, can be chemically conjugated to, or expressed as, fusion proteins with other proteins.

APP<sup>695</sup>, APP<sup>751</sup>, and APP<sup>770</sup> refer, respectively, to the 695, 751, and 770 amino acid residue long polypeptides encoded by the human APP gene. See Kang et al., *Nature* 325, 773 (1987); Ponte et al., *Nature* 331, 525 (1988); and Kitaguchi et al., *Nature* 331, 530 (1988). Amino acids within the human amyloid precursor protein (APP) are assigned numbers according to the sequence of the APP770 isoform. Terms such as A $\beta$ 39, A $\beta$ 40, A $\beta$ 41, A $\beta$ 42 and A $\beta$ 43 refer to an A $\beta$  peptide containing amino acid residues 1-39, 1-40, 1-41, 1-42 and 1-43.



The term "epitope" or "antigenic determinant" refers to a site on an antigen to which B and/or T cells respond. B-cell epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., *Epitope Mapping Protocols in Methods in Molecular Biology*, Vol. 66, Glenn E. Morris, Ed. (1996). Antibodies that recognize the same epitope can be identified in a simple immunoassay showing the ability of one antibody to block the binding of another antibody to a target antigen. T-cells recognize continuous epitopes of about nine amino acids for CD8 cells or about 13-15 amino acids for CD4 cells. T cells that recognize the epitope can be identified by *in vitro* assays that measure antigen-dependent proliferation, as determined by <sup>3</sup>H-thymidine incorporation by primed T cells in response to an epitope (Burke et al., *J. Inf. Dis.* 170, 1110-19 (1994)), by antigen-dependent killing (cytotoxic T lymphocyte assay, Tigges et al., *J. Immunol.* 156, 3901-3910) or by cytokine secretion.

The term "immunological" or "immune" response is the development of a beneficial humoral (antibody mediated) and/or a cellular (mediated by antigen-specific T cells or their secretion products) response directed against an amyloid peptide in a recipient patient. Such a response can be an active response induced by administration of immunogen or a passive response induced by administration of antibody or primed T-cells. A cellular immune response is elicited by the presentation of polypeptide epitopes in association with Class I or Class II MHC molecules to activate antigen-specific CD4<sup>+</sup> T helper cells and/or CD8<sup>+</sup> cytotoxic T cells. The response may also involve activation of monocytes, macrophages, NK

cells, basophils, dendritic cells, astrocytes, microglia cells, eosinophils or other components of innate immunity. The presence of a cell-mediated immunological response can be determined by proliferation assays (CD4<sup>+</sup> T cells) or CTL (cytotoxic T lymphocyte) assays (see Burke, supra; Tigges, supra). The relative contributions of humoral and cellular responses to the protective or therapeutic effect of an immunogen can be distinguished by separately isolating IgG and T-cells from an immunized syngeneic animal and measuring protective or therapeutic effect in a second subject.

An "immunogenic agent" or "immunogen" is capable of inducing an immunological response against itself on administration to a patient, optionally in conjunction with an adjuvant.

The term "naked polynucleotide" refers to a polynucleotide not complexed with colloidal materials. Naked polynucleotides are sometimes cloned in a plasmid vector.

The term "adjuvant" refers to a compound that when administered in conjunction with an antigen augments the immune response to the antigen, but when administered alone does not generate an immune response to the antigen. Adjuvants can augment an immune response by several mechanisms including lymphocyte recruitment, stimulation of B and/or T cells, and stimulation of macrophages.

The term "patient" includes human and other mammalian subjects that receive either prophylactic or therapeutic treatment.

Disaggregated or monomeric A $\beta$  means soluble, monomeric peptide units of A $\beta$ . One method to prepare monomeric A $\beta$  is to dissolve lyophilized peptide in neat DMSO with sonication. The resulting solution is centrifuged to remove any nonsoluble particulates. Aggregated A $\beta$  is a mixture of oligomers in which the monomeric units are held together by noncovalent bonds.

Compositions or methods "comprising" one or more recited elements may include other elements not specifically recited. For example, a composition that comprises A $\beta$  peptide

encompasses both an isolated A $\beta$  peptide and A $\beta$  peptide as a component of a larger polypeptide sequence.

## DETAILED DESCRIPTION

### I. General

5       The invention provides pharmaceutical compositions and methods for prophylactic and therapeutic treatment of diseases characterized by accumulation of amyloid deposits. Amyloid deposits comprise a peptide aggregated to an insoluble mass. The nature of the peptide varies in different diseases but in  
10       most cases, the aggregate has a  $\beta$ -pleated sheet structure and stains with Congo Red dye. Diseases characterized by amyloid deposits include Alzheimer's disease (AD), both late and early onset. In both diseases, the amyloid deposit comprises a peptide termed A $\beta$ , which accumulates in the brain of affected  
15       individuals. Examples of some other diseases characterized by amyloid deposits are SAA amyloidosis, hereditary Icelandic syndrome, multiple myeloma, and spongiform encephalopathies, including mad cow disease, Creutzfeldt Jakob disease, sheep scrapie, and mink spongiform encephalopathy (see Weissmann et  
20       al., *Curr. Opin. Neurobiol.* 7, 695-700 (1997); Smits et al., *Veterinary Quarterly* 19, 101-105 (1997); Nathanson et al., *Am. J. Epidemiol.* 145, 959-969 (1997)). The peptides forming the aggregates in these diseases are serum amyloid A, cystatin C, IgG kappa light chain respectively for the first three, and  
25       prion protein for the others.

### II. Therapeutic Agents

#### 1. Alzheimer's Disease

Therapeutic agents for use in the present invention induce an immune response against A $\beta$  peptide. These agents include  
30       A $\beta$  peptide itself and variants thereof, analogs and mimetics of A $\beta$  peptide that induce and/or crossreact with antibodies to A $\beta$  peptide, and antibodies or T-cells reactive with A $\beta$  peptide. Induction of an immune response can be active as when an immunogen is administered to induce antibodies or T-  
35       cells reactive with A $\beta$  in a patient, or passive, as when an antibody is administered that itself binds to A $\beta$  in patient.

$A\beta$ , also known as  $\beta$ -amyloid peptide, or A4 peptide (see US 4,666,829; Glenner & Wong, *Biochem. Biophys. Res. Commun.* 120, 1131 (1984)), is a peptide of 39-43 amino acids, which is the principal component of characteristic plaques of Alzheimer's disease.  $A\beta$  is generated by processing of a larger protein APP by two enzymes, termed  $\beta$  and  $\gamma$  secretases (see Hardy, *TINS* 20, 154 (1997)). Known mutations in APP associated with Alzheimer's disease occur proximate to the site of  $\beta$  or  $\gamma$  secretase, or within  $A\beta$ . For example, position 717 is proximate to the site of  $\gamma$ -secretase cleavage of APP in its processing to  $A\beta$ , and positions 670/671 are proximate to the site of  $\beta$ -secretase cleavage. It is believed that the mutations cause AD disease by interacting with the cleavage reactions by which  $A\beta$  is formed so as to increase the amount of the 42/43 amino acid form of  $A\beta$  generated.

$A\beta$  has the unusual property that it can fix and activate both classical and alternate complement cascades. In particular, it binds to C1q and ultimately to C3bi. This association facilitates binding to macrophages leading to activation of B cells. In addition, C3bi breaks down further and then binds to CR2 on B cells in a T cell dependent manner leading to a 10,000 increase in activation of these cells. This mechanism causes  $A\beta$  to generate an immune response in excess of that of other antigens.

The therapeutic agent used in the claimed methods can be any of the naturally occurring forms of  $A\beta$  peptide, and particularly the human forms (i.e.,  $A\beta$ 39,  $A\beta$ 40,  $A\beta$ 41,  $A\beta$ 42 or  $A\beta$ 43). The sequences of these peptides and their relationship to the APP precursor are illustrated by Fig. 1 of Hardy et al., *TINS* 20, 155-158 (1997). For example,  $A\beta$ 42 has the sequence:

H<sub>2</sub>N-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala-OH.

$A\beta$ 41,  $A\beta$ 40 and  $A\beta$ 39 differ from  $A\beta$ 42 by the omission of Ala, Ala-Ile, and Ala-Ile-Val respectively from the C-terminal end.  $A\beta$ 43 differs from  $A\beta$ 42 by the presence of a threonine residue

at the C-terminus. The therapeutic agent can also be an active fragment or analog of a natural A $\beta$  peptide that contains an epitope that induces a similar protective or therapeutic immune response on administration to a human.

5 Immunogenic fragments typically have a sequence of at least 3, 5, 6, 10 or 20 contiguous amino acids from a natural peptide. Immunogenic fragments include A $\beta$ 1-5, 1-6, 1-12, 13-28, 17-28, 25-25, 35-40 and 35-42. Fragments from the N-terminal half of A $\beta$  are preferred in some methods. Analogs include allelic,  
10 species and induced variants. Analogs typically differ from naturally occurring peptides at one or a few positions, often by virtue of conservative substitutions. Analogs typically exhibit at least 80 or 90% sequence identity with natural peptides. Some analogs also include unnatural amino acids or  
15 modifications of N or C terminal amino acids. Examples of unnatural amino acids are  $\alpha,\alpha$ -disubstituted amino acids, N-alkyl amino acids, lactic acid, 4-hydroxyproline,  $\gamma$ -carboxyglutamate,  $\epsilon$ -N,N,N-trimethyllysine,  $\epsilon$ -N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine,  $\omega$ -N-methylarginine.  
20 Fragments and analogs can be screened for prophylactic or therapeutic efficacy in transgenic animal models as described below.

A $\beta$ , its fragments, analogs and other amyloidogenic peptides  
25 can be synthesized by solid phase peptide synthesis or recombinant expression, or can be obtained from natural sources. Automatic peptide synthesizers are commercially available from numerous suppliers, such as Applied Biosystems, Foster City, California. Recombinant expression can be in  
30 bacteria, such as E. coli, yeast, insect cells or mammalian cells. Procedures for recombinant expression are described by Sambrook et al., *Molecular Cloning: A Laboratory Manual* (C.S.H.P. Press, NY 2d ed., 1989). Some forms of A $\beta$  peptide are also available commercially (e.g., American Peptides  
35 Company, Inc., Sunnyvale, CA and California Peptide Research, Inc. Napa, CA).

Therapeutic agents also include longer polypeptides that include, for example, an A $\beta$  peptide, active fragment or analog

together with other amino acids. For example, A $\beta$  peptide can be present as intact APP protein or a segment thereof, such as the C-100 fragment that begins at the N-terminus of A $\beta$  and continues to the end of APP. Such polypeptides can be  
5 screened for prophylactic or therapeutic efficacy in animal models as described below. The A $\beta$  peptide, analog, active fragment or other polypeptide can be administered in associated form (i.e., as an amyloid peptide) or in dissociated form. Therapeutic agents also include multimers  
10 of monomeric immunogenic agents.

In a further variation, an immunogenic peptide, such as A $\beta$ , can be presented as a viral or bacterial vaccine. A nucleic acid encoding the immunogenic peptide is incorporated into a genome or episome of the virus or bacteria. Optionally, the  
15 nucleic acid is incorporated in such a manner that the immunogenic peptide is expressed as a secreted protein or as a fusion protein with an outersurface protein of a virus or a transmembrane protein of a bacteria so that the peptide is displayed. Viruses or bacteria used in such methods should  
20 be nonpathogenic or attenuated. Suitable viruses include adenovirus, HSV, vaccinia and fowl pox. Fusion of an immunogenic peptide to HBsAg of HBV is particularly suitable. Therapeutic agents also include peptides and other compounds that do not necessarily have a significant amino acid sequence  
25 similarity with A $\beta$  but nevertheless serve as mimetics of A $\beta$  and induce a similar immune response. For example, any peptides and proteins forming  $\beta$ -pleated sheets can be screened for suitability. Anti-idiotypic antibodies against monoclonal antibodies to A $\beta$  or other amyloidogenic peptides can also be  
30 used. Such anti-Id antibodies mimic the antigen and generate an immune response to it (see *Essential Immunology* (Roit ed., Blackwell Scientific Publications, Palo Alto, 6th ed.), p. 181).

Random libraries of peptides or other compounds can also be  
35 screened for suitability. Combinatorial libraries can be produced for many types of compounds that can be synthesized in a step-by-step fashion. Such compounds include polypeptides, beta-turn mimetics, polysaccharides,

phospholipids, hormones, prostaglandins, steroids, aromatic compounds, heterocyclic compounds, benzodiazepines, oligomeric N-substituted glycines and oligocarbamates. Large combinatorial libraries of the compounds can be constructed by the encoded synthetic libraries (ESL) method described in Affymax, WO 95/12608, Affymax, WO 93/06121, Columbia University, WO 94/08051, Pharmacopeia, WO 95/35503 and Scripps, WO 95/30642 (each of which is incorporated by reference for all purposes). Peptide libraries can also be generated by phage display methods. See, e.g., Devlin, WO 91/18980.

Combinatorial libraries and other compounds are initially screened for suitability by determining their capacity to bind to antibodies or lymphocytes (B or T) known to be specific for A $\beta$  or other amyloidogenic peptides. For example, initial screens can be performed with any polyclonal sera or monoclonal antibody to A $\beta$  or other amyloidogenic peptide. Compounds identified by such screens are then further analyzed for capacity to induce antibodies or reactive lymphocytes to A $\beta$  or other amyloidogenic peptide. For example, multiple dilutions of sera can be tested on microtiter plates that have been precoated with A $\beta$  peptide and a standard ELISA can be performed to test for reactive antibodies to A $\beta$ . Compounds can then be tested for prophylactic and therapeutic efficacy in transgenic animals predisposed to an amyloidogenic disease, as described in the Examples. Such animals include, for example, mice bearing a 717 mutation of APP described by Games et al., supra, and mice bearing a Swedish mutation of APP such as described by McConlogue et al., US 5,612,486 and Hsiao et al., *Science* 274, 99 (1996); Staufenbiel et al., *Proc. Natl. Acad. Sci. USA* 94, 13287-13292 (1997); Sturchler-Pierrat et al., *Proc. Natl. Acad. Sci. USA* 94, 13287-13292 (1997); Borchelt et al., *Neuron* 19, 939-945 (1997)). The same screening approach can be used on other potential agents such as fragments of A $\beta$ , analogs of A $\beta$  and longer peptides including A $\beta$ , described above.

Therapeutic agents of the invention also include antibodies that specifically bind to A $\beta$ . Such antibodies can be

monoclonal or polyclonal. Some such antibodies bind specifically to the aggregated form of A $\beta$  without binding to the dissociated form. Some bind specifically to the dissociated form without binding to the aggregated form. Some  
5 bind to both aggregated and dissociated forms. The production of non-human monoclonal antibodies, e.g., murine or rat, can be accomplished by, for example, immunizing the animal with A $\beta$ . See Harlow & Lane, *Antibodies, A Laboratory Manual* (CSHP NY, 1988) (incorporated by reference for all purposes). Such  
10 an immunogen can be obtained from a natural source, by peptides synthesis or by recombinant expression.

Humanized forms of mouse antibodies can be generated by linking the CDR regions of non-human antibodies to human constant regions by recombinant DNA techniques. See Queen et  
15 al., *Proc. Natl. Acad. Sci. USA* 86, 10029-10033 (1989) and WO 90/07861 (incorporated by reference for all purposes).

Human antibodies can be obtained using phage-display methods. See, e.g., Dower et al., WO 91/17271; McCafferty et al., WO 92/01047. In these methods, libraries of phage are  
20 produced in which members display different antibodies on their outersurfaces. Antibodies are usually displayed as Fv or Fab fragments. Phage displaying antibodies with a desired specificity are selected by affinity enrichment to A $\beta$ , or fragments thereof. Human antibodies against A $\beta$  can also be  
25 produced from non-human transgenic mammals having transgenes encoding at least a segment of the human immunoglobulin locus and an inactivated endogenous immunoglobulin locus. See, e.g., Lonberg et al., WO93/12227 (1993); Kucherlapati, WO 91/10741 (1991) (each of which is incorporated by reference in  
30 its entirety for all purposes). Human antibodies can be selected by competitive binding experiments, or otherwise, to have the same epitope specificity as a particular mouse antibody. Such antibodies are particularly likely to share the useful functional properties of the mouse antibodies.  
35 Human polyclonal antibodies can also be provided in the form of serum from humans immunized with an immunogenic agent. Optionally, such polyclonal antibodies can be concentrated by



affinity purification using A $\beta$  or other amyloid peptide as an affinity reagent.

Human or humanized antibodies can be designed to have IgG, IgD, IgA and IgE constant region, and any isotype, including IgG1, IgG2, IgG3 and IgG4. Antibodies can be expressed as tetramers containing two light and two heavy chains, as separate heavy chains, light chains, as Fab, Fab' F(ab')<sub>2</sub>, and Fv, or as single chain antibodies in which heavy and light chain variable domains are linked through a spacer.

Therapeutic agents for use in the present methods also include T-cells that bind to A $\beta$  peptide. For example, T-cells can be activated against A $\beta$  peptide by expressing a human MHC class I gene and a human  $\beta$ -2-microglobulin gene from an insect cell line, whereby an empty complex is formed on the surface of the cells and can bind to A $\beta$  peptide. T-cells contacted with the cell line become specifically activated against the peptide. See Peterson et al., US 5,314,813. Insect cell lines expressing an MHC class II antigen can similarly be used to activate CD4 T cells.

## 2. Other Diseases

The same or analogous principles determine production of therapeutic agents for treatment of other amyloidogenic diseases. In general, the agents noted above for use in treatment of Alzheimer's disease can also be used for treatment early onset Alzheimer's disease associated with Down's syndrome. In mad cow disease, prion peptide, active fragments, and analogs, and antibodies to prion peptide are used in place of A $\beta$  peptide, active fragments, analogs and antibodies to A $\beta$  peptide in treatment of Alzheimer's disease. In treatment of multiple myeloma, IgG light chain and analogs and antibodies thereto are used, and so forth in other diseases.

## 3. Carrier Proteins

Some agents for inducing an immune response contain the appropriate epitope for inducing an immune response against amyloid deposits but are too small to be immunogenic. In this

situation, a peptide immunogen can be linked to a suitable carrier to help elicit an immune response. Suitable carriers include serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, tetanus toxoid, or a toxoid from other pathogenic bacteria, such as diphtheria, *E. coli*, cholera, or *H. pylori*, or an attenuated toxin derivative. Other carriers for stimulating or enhancing an immune response include cytokines such as IL-1, IL-1  $\alpha$  and  $\beta$  peptides, IL-2,  $\gamma$ INF, IL-10, GM-CSF, and chemokines, such as MIP1 $\alpha$  and  $\beta$  and RANTES. Immunogenic agents can also be linked to peptides that enhance transport across tissues, as described in O'Mahony, WO 97/17613 and WO 97/17614.

Immunogenic agents can be linked to carriers by chemical crosslinking. Techniques for linking an immunogen to a carrier include the formation of disulfide linkages using N-succinimidyl-3-(2-pyridyl-thio) propionate (SPDP) and succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) (if the peptide lacks a sulfhydryl group, this can be provided by addition of a cysteine residue). These reagents create a disulfide linkage between themselves and peptide cysteine residues on one protein and an amide linkage through the  $\epsilon$ -amino on a lysine, or other free amino group in other amino acids. A variety of such disulfide/amide-forming agents are described by *Immun. Rev.* 62, 185 (1982). Other bifunctional coupling agents form a thioether rather than a disulfide linkage. Many of these thio-ether-forming agents are commercially available and include reactive esters of 6-maleimidocaproic acid, 2-bromoacetic acid, and 2-iodoacetic acid, 4-(N-maleimido-methyl)cyclohexane-1-carboxylic acid. The carboxyl groups can be activated by combining them with succinimide or 1-hydroxyl-2-nitro-4-sulfonic acid, sodium salt.

Immunogenic peptides can also be expressed as fusion proteins with carriers. The immunogenic peptide can be linked at the amino terminus, the carboxyl terminus, or internally to the carrier. Optionally, multiple repeats of the immunogenic peptide can be present in the fusion protein.

#### 4. Nucleic Acid Encoding Immunogens

Immune responses against amyloid deposits can also be induced by administration of nucleic acids encoding A $\beta$  peptide or other peptide immunogens. Such nucleic acids can be DNA or RNA. A nucleic acid segment encoding the immunogen is typically linked to regulatory elements, such as a promoter and enhancer, that allow expression of the DNA segment in the intended target cells of a patient. For expression in blood cells, as is desirable for induction of an immune response, promoter and enhancer elements from light or heavy chain immunoglobulin genes or the CMV major intermediate early promoter and enhancer are suitable to direct expression. The linked regulatory elements and coding sequences are often cloned into a vector.

A number of viral vector systems are available including retroviral systems (see, e.g., Lawrie and Tumin, *Cur. Opin. Genet. Develop.* 3, 102-109 (1993)); adenoviral vectors (see, e.g., Bett et al., *J. Virol.* 67, 5911 (1993)); adeno-associated virus vectors (see, e.g., Zhou et al., *J. Exp. Med.* 179, 1867 (1994)), viral vectors from the pox family including vaccinia virus and the avian pox viruses, viral vectors from the alpha virus genus such as those derived from Sindbis and Semliki Forest Viruses (see, e.g., Dubensky et al., *J. Virol.* 70, 508-519 (1996)), and papillomaviruses (Ohe et al., *Human Gene Therapy* 6, 325-333 (1995); Woo et al., WO 94/12629 and Xiao & Brandsma, *Nucleic Acids. Res.* 24, 2630-2622 (1996)).

DNA encoding an immunogen, or a vector containing the same, can be packaged into liposomes. Suitable lipids and related analogs are described by US 5,208,036, 5,264,618, 5,279,833 and 5,283,185. Vectors and DNA encoding an immunogen can also be adsorbed to or associated with particulate carriers, examples of which include polymethyl methacrylate polymers and polylactides and poly(lactide-co-glycolides), see, e.g., McGee et al., *J. Micro Encap.* (1996).

Gene therapy vectors or naked DNA can be delivered *in vivo* by administration to an individual patient, typically by systemic administration (e.g., intravenous, intraperitoneal, nasal, gastric, intradermal, intramuscular, subdermal, or

intracranial infusion) or topical application (see e.g.,  
US 5,399,346). DNA can also be administered using a gene  
gun. See Xiao & Brandsma, *supra*. The DNA encoding an  
immunogen is precipitated onto the surface of microscopic  
5 metal beads. The microprojectiles are accelerated with a  
shock wave or expanding helium gas, and penetrate tissues to a  
depth of several cell layers. For example, The Accel<sup>TM</sup> Gene  
Delivery Device manufactured by Agacetus, Inc. Middleton WI is  
suitable. Alternatively, naked DNA can pass through skin into  
10 the blood stream simply by spotting the DNA onto skin with  
chemical or mechanical irritation (see WO 95/05853).

In a further variation, vectors encoding immunogens can be  
delivered to cells *ex vivo*, such as cells explanted from an  
individual patient (e.g., lymphocytes, bone marrow aspirates,  
15 tissue biopsy) or universal donor hematopoietic stem cells,  
followed by reimplantation of the cells into a patient,  
usually after selection for cells which have incorporated the  
vector.

### III. Patients Amenable to Treatment

20 Patients amenable to treatment include individuals at risk  
of disease but not showing symptoms, as well as patients  
presently showing symptoms. In the case of Alzheimer's  
disease, virtually anyone is at risk of suffering from  
Alzheimer's disease if he or she lives long enough.  
25 Therefore, the present methods can be administered  
prophylactically to the general population without any  
assessment of the risk of the subject patient. The present  
methods are especially useful for individuals who do have a  
known genetic risk of Alzheimer's disease. Such individuals  
30 include those having relatives who have experienced this  
disease, and those whose risk is determined by analysis of  
genetic or biochemical markers. Genetic markers of risk  
toward Alzheimer's disease include mutations in the APP gene,  
particularly mutations at position 717 and positions 670 and  
35 671 referred to as the Hardy and Swedish mutations  
respectively (see Hardy, *TINS*, *supra*). Other markers of risk  
are mutations in the presenilin genes, PS1 and PS2, and ApoE4,

family history of AD, hypercholesterolemia or atherosclerosis. Individuals presently suffering from Alzheimer's disease can be recognized from characteristic dementia, as well as the presence of risk factors described above. In addition, a number of diagnostic tests are available for identifying individuals who have AD. These include measurement of CSF tau and A $\beta$ 42 levels. Elevated tau and decreased A $\beta$ 42 levels signify the presence of AD. Individuals suffering from Alzheimer's disease can also be diagnosed by MMSE or ADRDA criteria as discussed in the Examples section.

In asymptomatic patients, treatment can begin at any age (e.g., 10, 20, 30). Usually, however, it is not necessary to begin treatment until a patient reaches 40, 50, 60 or 70. Treatment typically entails multiple dosages over a period of time. Treatment can be monitored by assaying antibody, or activated T-cell or B-cell responses to the therapeutic agent (e.g., A $\beta$  peptide) over time. If the response falls, a booster dosage is indicated. In the case of potential Down's syndrome patients, treatment can begin antenatally by administering therapeutic agent to the mother or shortly after birth.

#### IV. Treatment Regimes

In prophylactic applications, pharmaceutical compositions or medicants are administered to a patient susceptible to, or otherwise at risk of, a particular disease in an amount sufficient to eliminate or reduce the risk or delay the outset of the disease. In therapeutic applications, compositions or medicants are administered to a patient suspected of, or already suffering from such a disease in an amount sufficient to cure, or at least partially arrest, the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as a therapeutically- or pharmaceutically-effective dose. In both prophylactic and therapeutic regimes, agents are usually administered in several dosages until a sufficient immune response has been achieved. Typically, the immune response is monitored and

repeated dosages are given if the immune response starts to fade.

Effective doses of the compositions of the present invention, for the treatment of the above described conditions vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and whether treatment is prophylactic or therapeutic. Usually, the patient is a human, but in some diseases, such as mad cow disease, the patient can be a nonhuman mammal, such as a bovine. Treatment dosages need to be titrated to optimize safety and efficacy. The amount of immunogen depends on whether adjuvant is also administered, with higher dosages being required in the absence of adjuvant. The amount of an immunogen for administration sometimes varies from 1  $\mu$ g-500  $\mu$ g per patient and more usually from 5-500  $\mu$ g per injection for human administration. Occasionally, a higher dose of 1-2 mg per injection is used. Typically about 10, 20, 50 or 100  $\mu$ g is used for each human injection. The timing of injections can vary significantly from once a day, to once a year, to once a decade. On any given day that a dosage of immunogen is given, the dosage is greater than 1  $\mu$ g/patient and usually greater than 10  $\mu$ g/patient if adjuvant is also administered, and greater than 10  $\mu$ g/patient and usually greater than 100  $\mu$ g/patient in the absence of adjuvant. A typical regimen consists of an immunization followed by booster injections at 6 weekly intervals. Another regimen consists of an immunization followed by booster injections 1, 2 and 12 months later. Another regimen entails an injection every two months for life. Alternatively, booster injections can be on an irregular basis as indicated by monitoring of immune response. For passive immunization with an antibody, the dosage ranges from about 0.0001 to 100 mg/kg, and more usually 0.01 to 5 mg/kg of the host body weight. Doses for nucleic acids encoding immunogens range from about 10 ng to 1 g, 100 ng to 100 mg, 1  $\mu$ g to 10 mg, or 30-300  $\mu$ g DNA per patient. Doses

for infectious viral vectors vary from  $10^{-10}$  to  $10^9$ , or more, virions per dose.

Agents for inducing an immune response can be administered by parenteral, topical, intravenous, oral, subcutaneous, intraperitoneal, intranasal or intramuscular means for prophylactic and/or therapeutic treatment. The most typical route of administration is subcutaneous although others can be equally effective. The next most common is intramuscular injection. This type of injection is most typically performed in the arm or leg muscles. Intravenous injections as well as intraperitoneal injections, intraarterial, intracranial, or intradermal injections are also effective in generating an immune response. In some methods, agents are injected directly into a particular tissue where deposits have accumulated.

Agents of the invention can optionally be administered in combination with other agents that are at least partly effective in treatment of amyloidogenic disease. In the case of Alzheimer's and Down's syndrome, in which amyloid deposits occur in the brain, agents of the invention can also be administered in conjunction with other agents that increase passage of the agents of the invention across the blood-brain barrier.

Immunogenic agents of the invention, such as peptides, are sometimes administered in combination with an adjuvant. A variety of adjuvants can be used in combination with a peptide, such as A $\beta$ , to elicit an immune response. Preferred adjuvants augment the intrinsic response to an immunogen without causing conformational changes in the immunogen that affect the qualitative form of the response. Preferred adjuvants include alum, 3 De-O-acylated monophosphoryl lipid A (MPL) (see GB 2220211). QS21 is a triterpene glycoside or saponin isolated from the bark of the Quillaja Saponaria Molina tree found in South America (see Kensil et al., in *Vaccine Design: The Subunit and Adjuvant Approach* (eds. Powell & Newman, Plenum Press, NY, 1995); US Patent No. 5,057,540). Other adjuvants are oil in water emulsions (such as squalene or peanut oil), optionally in combination with immune

stimulants, such as monophosphoryl lipid A (see Stoute et al., *N. Engl. J. Med.* 336, 86-91 (1997)). Another adjuvant is CpG (*Bioworld Today*, Nov. 15, 1998). Alternatively, A $\beta$  can be coupled to an adjuvant. For example, a lipopeptide version of A $\beta$  can be prepared by coupling palmitic acid or other lipids directly to the N-terminus of A $\beta$  as described for hepatitis B antigen vaccination (Livingston, *J. Immunol.* 159, 1383-1392 (1997)). However, such coupling should not substantially change the conformation of A $\beta$  so as to affect the nature of the immune response thereto. Adjuvants can be administered as a component of a therapeutic composition with an active agent or can be administered separately, before, concurrently with, or after administration of the therapeutic agent.

A preferred class of adjuvants is aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate. Such adjuvants can be used with or without other specific immunostimulating agents such as MPL or 3-DMP, QS21, polymeric or monomeric amino acids such as polyglutamic acid or polylysine. Another class of adjuvants is oil-in-water emulsion formulations. Such adjuvants can be used with or without other specific immunostimulating agents such as muramyl peptides (e.g., N-acetylmuramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE), N-acetylglucosaminyl-N-acetylmuramyl-L-Al-D-isoglu-L-Ala-dipalmitoxy propylamide (DTP-DPP) theramide<sup>TM</sup>), or other bacterial cell wall components. Oil-in-water emulsions include (a) MF59 (WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton MA), (b) SAF, containing 10% Squalene, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP, either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribi<sup>TM</sup> adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT)



containing 2% squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™).

5 Another class of preferred adjuvants is saponin adjuvants, such as Stimulon™ (QS21, Aquila, Worcester, MA) or particles generated therefrom such as ISCOMs (immunostimulating complexes) and ISCOMATRIX. Other adjuvants include Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant  
10 (IFA). Other adjuvants include cytokines, such as interleukins (IL-1, IL-2, and IL-12), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF).

An adjuvant can be administered with an immunogen as a single composition, or can be administered before, concurrent  
15 with or after administration of the immunogen. Immunogen and adjuvant can be packaged and supplied in the same vial or can be packaged in separate vials and mixed before use. Immunogen and adjuvant are typically packaged with a label indicating the intended therapeutic application. If immunogen and  
20 adjuvant are packaged separately, the packaging typically includes instructions for mixing before use. The choice of an adjuvant and/or carrier depends on the stability of the vaccine containing the adjuvant, the route of administration, the dosing schedule, the efficacy of the adjuvant for the  
25 species being vaccinated, and, in humans, a pharmaceutically acceptable adjuvant is one that has been approved or is approvable for human administration by pertinent regulatory bodies. For example, Complete Freund's adjuvant is not suitable for human administration. Alum, MPL and QS21 are  
30 preferred. Optionally, two or more different adjuvants can be used simultaneously. Preferred combinations include alum with MPL, alum with QS21, MPL with QS21, and alum, QS21 and MPL together. Also, Incomplete Freund's adjuvant can be used  
(Chang et al., *Advanced Drug Delivery Reviews* 32, 173-186  
35 (1998)), optionally in combination with any of alum, QS21, and MPL and all combinations thereof.

Agents of the invention are often administered as pharmaceutical compositions comprising an active therapeutic

agent, i.e., and a variety of other pharmaceutically acceptable components. See *Remington's Pharmaceutical Science* (15th ed., Mack Publishing Company, Easton, Pennsylvania, 1980). The preferred form depends on the intended mode of administration and therapeutic application. The compositions can also include, depending on the formulation desired, pharmaceutically-acceptable, non-toxic carriers or diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the combination. Examples of such diluents are distilled water, physiological phosphate-buffered saline, Ringer's solutions, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation may also include other carriers, adjuvants, or nontoxic, nontherapeutic, nonimmunogenic stabilizers and the like. However, some reagents suitable for administration to animals, such as Complete Freund's adjuvant are not typically included in compositions for human use.

Pharmaceutical compositions can also include large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids and copolymers (such as latex functionalized sepharose, agarose, cellulose, and the like), polymeric amino acids, amino acid copolymers, and lipid aggregates (such as oil droplets or liposomes). Additionally, these carriers can function as immunostimulating agents (i.e., adjuvants).

For parenteral administration, agents of the invention can be administered as injectable dosages of a solution or suspension of the substance in a physiologically acceptable diluent with a pharmaceutical carrier which can be a sterile liquid such as water oils, saline, glycerol, or ethanol. Additionally, auxiliary substances, such as wetting or emulsifying agents, surfactants, pH buffering substances and the like can be present in compositions. Other components of pharmaceutical compositions are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, and mineral oil. In general, glycols such as

propylene glycol or polyethylene glycol are preferred liquid carriers, particularly for injectable solutions.

Typically, compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared. The preparation also can be emulsified or encapsulated in liposomes or micro particles such as polylactide, polyglycolide, or copolymer for enhanced adjuvant effect, as discussed above (see Langer, *Science* 249, 1527 (1990) and Hanes, *Advanced Drug Delivery Reviews* 28, 97-119 (1997)). The agents of this invention can be administered in the form of a depot injection or implant preparation which can be formulated in such a manner as to permit a sustained or pulsatile release of the active ingredient.

Additional formulations suitable for other modes of administration include oral, intranasal, and pulmonary formulations, suppositories, and transdermal applications.

For suppositories, binders and carriers include, for example, polyalkylene glycols or triglycerides; such suppositories can be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%. Oral formulations include excipients, such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, and magnesium carbonate. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10%-95% of active ingredient, preferably 25%-70%.

Topical application can result in transdermal or intradermal delivery. Topical administration can be facilitated by co-administration of the agent with cholera toxin or detoxified derivatives or subunits thereof or other similar bacterial toxins (See Glenn et al., *Nature* 391, 851 (1998)). Co-administration can be achieved by using the components as a mixture or as linked molecules obtained by chemical crosslinking or expression as a fusion protein.

Alternatively, transdermal delivery can be achieved using a skin path or using transferosomes (Paul et al., *Eur. J.*

*Immunol.* 25, 3521-24 (1995); Cevc et al., *Biochem. Biophys. Acta* 1368, 201-15 (1998)).

#### V. Methods of Diagnosis

The invention provides methods of detecting an immune response against A $\beta$  peptide in a patient suffering from or susceptible to Alzheimer's disease. The methods are particularly useful for monitoring a course of treatment being administered to a patient. The methods can be used to monitor both therapeutic treatment on symptomatic patients and prophylactic treatment on asymptomatic patients.

Some methods entail determining a baseline value of an immune response in a patient before administering a dosage of agent, and comparing this with a value for the immune response after treatment. A significant increase (i.e., greater than the typical margin of experimental error in repeat measurements of the same sample, expressed as one standard deviation from the mean of such measurements) in value of the immune response signals a positive treatment outcome (i.e., that administration of the agent has achieved or augmented an immune response). If the value for immune response does not change significantly, or decreases, a negative treatment outcome is indicated. In general, patients undergoing an initial course of treatment with an agent are expected to show an increase in immune response with successive dosages, which eventually reaches a plateau. Administration of agent is generally continued while the immune response is increasing. Attainment of the plateau is an indicator that the administered treatment can be discontinued or reduced in dosage or frequency.

In other methods, a control value (i.e., a mean and standard deviation) of immune response is determined for a control population. Typically the individuals in the control population have not received prior treatment. Measured values of immune response in a patient after administering a therapeutic agent are then compared with the control value. A significant increase relative to the control value (e.g., greater than one standard deviation from the mean) signals a

positive treatment outcome. A lack of significant increase or a decrease signals a negative treatment outcome.

Administration of agent is generally continued while the immune response is increasing relative to the control value.

5 As before, attainment of a plateau relative to control values in an indicator that the administration of treatment can be discontinued or reduced in dosage or frequency.

10 In other methods, a control value of immune response (e.g., a mean and standard deviation) is determined from a control population of individuals who have undergone treatment with a therapeutic agent and whose immune responses have plateaued in response to treatment. Measured values of immune response in a patient are compared with the control value. If the measured level in a patient is not significantly different  
15 (e.g., more than one standard deviation) from the control value, treatment can be discontinued. If the level in a patient is significantly below the control value, continued administration of agent is warranted. If the level in the patient persists below the control value, then a change in  
20 treatment regime, for example, use of a different adjuvant may be indicated.

In other methods, a patient who is not presently receiving treatment but has undergone a previous course of treatment is monitored for immune response to determine whether a  
25 resumption of treatment is required. The measured value of immune response in the patient can be compared with a value of immune response previously achieved in the patient after a previous course of treatment. A significant decrease relative to the previous measurement (i.e., greater than a typical  
30 margin of error in repeat measurements of the same sample) is an indication that treatment can be resumed. Alternatively, the value measured in patient can be compared with a control value (mean plus standard deviation) determined in population of patients after undergoing a course of treatment.

35 Alternatively, the measured value in a patient can be compared with a control value in populations of prophylactically treated patients who remain free of symptoms of disease, or populations of therapeutically treated patients who show

amelioration of disease characteristics. In all of these cases, a significant decrease relative to the control level (i.e., more than a standard deviation) is an indicator that treatment should be resumed in a patient.

5 The tissue sample for analysis is typically blood, plasma, serum, mucus or cerebral spinal fluid from the patient. The sample is analyzed for indicia of an immune response to any form of A $\beta$  peptide, typically A $\beta$ 42. The immune response can be determined from the presence of, e.g., antibodies or T-  
10 cells that specifically bind to A $\beta$  peptide. ELISA methods of detecting antibodies specific to A $\beta$  are described in the Examples section. Methods of detecting reactive T-cells have been described above (see Definitions).

The invention further provides diagnostic kits for  
15 performing the diagnostic methods described above. Typically, such kits contain an agent that specifically binds to antibodies to A $\beta$  or reacts with T-cells specific for A $\beta$ . The kit can also include a label. For detection of antibodies to A $\beta$ , the label is typically in the form of labelled anti-  
20 idiotypic antibodies. For detection of antibodies, the agent can be supplied prebound to a solid phase, such as to the wells of a microtiter dish. For detection of reactive T-cells, the label can be supplied as <sup>3</sup>H-thymidine to measure a proliferative response. Kits also typically contain labelling  
25 providing directions for use of the kit. The labelling may also include a chart or other correspondence regime correlating levels of measured label with levels of antibodies to A $\beta$  or T-cells reactive with A $\beta$ . The term labelling refers to any written or recorded material that is attached to, or  
30 otherwise accompanies a kit at any time during its manufacture, transport, sale or use. For example, the term labelling encompasses advertising leaflets and brochures, packaging materials, instructions, audio or video cassettes, computer discs, as well as writing imprinted directly on kits.

## 35 EXAMPLES

### I. Prophylactic Efficacy of A $\beta$ Against AD

These examples describe administration of A $\beta$ <sub>42</sub> peptide to transgenic mice overexpressing APP with a mutation at position 717 (APP<sub>717V→F</sub>) that predisposes them to develop Alzheimer's-like neuropathology. Production and characteristics of these mice (PDAPP mice) is described in Games et al., *Nature, supra*. These animals, in their heterozygote form, begin to deposit A $\beta$  at six months of age forward. By fifteen months of age they exhibit levels of A $\beta$  deposition equivalent to that seen in Alzheimer's disease. PDAPP mice were injected with aggregated A $\beta$ <sub>42</sub> (aggregated A $\beta$ <sub>42</sub>) or phosphate buffered saline. Aggregated A $\beta$ <sub>42</sub> was chosen because of its ability to induce antibodies to multiple epitopes of A $\beta$ .

## A. Methods

### 1. Source of Mice

Thirty PDAPP heterogenic female mice were randomly divided into the following groups: 10 mice to be injected with aggregated  $A\beta_{42}$  (one died in transit), 5 mice to be injected with PBS/adjuvant or PBS, and 10 uninjected controls. Five mice were injected with serum amyloid protein (SAP).

### 2. Preparation of Immunogens

Preparation of aggregated  $A\beta_{42}$ : two milligrams of  $A\beta_{42}$  (US Peptides Inc, lot K-42-12) was dissolved in 0.9 ml water and made up to 1 ml by adding 0.1 ml 10 x PBS. This was vortexed and allowed to incubate overnight 37° C, under which conditions the peptide aggregated. Any unused  $A\beta$  was stored as a dry lyophilized powder at -20° C until the next injection.

### 3. Preparation of Injections

100  $\mu$ g of aggregated  $A\beta_{42}$  in PBS per mouse was emulsified 1:1 with Complete Freund's adjuvant (CFA) in a final volume of 400  $\mu$ l emulsion for the first immunization, followed by a boost of the same amount of immunogen in Incomplete Freund's adjuvant (IFA) at 2 weeks. Two additional doses in IFA were given at monthly intervals. The subsequent immunizations were done at monthly intervals in 500  $\mu$ l of PBS. Injections were delivered intraperitoneally (i.p.).

PBS injections followed the same schedule and mice were injected with a 1:1 mix of PBS/ Adjuvant at 400  $\mu$ l per mouse, or 500  $\mu$ l of PBS per mouse. SAP injections likewise followed the same schedule using a dose of 100  $\mu$ g per injection.

### 4. Titration of Mouse Bleeds, Tissue Preparation and Immunohistochemistry

The above methods are described *infra* in General Materials and Methods.

## B. Results

PDAPP mice were injected with either aggregated  $A\beta_{42}$  (aggregated  $A\beta_{42}$ ), SAP peptides, or phosphate buffered saline.



A group of PDAPP mice were also left as uninjected, positive controls. The titers of the mice to aggregated  $A\beta_{42}$  were monitored every other month from the fourth boost until the mice were one year of age. Mice were sacrificed at 13 months. At all time points examined, eight of the nine aggregated  $A\beta_{42}$  mice developed a high antibody titer, which remained high throughout the series of injections (titers greater than 1/10000). The ninth mouse had a low, but measurable titer of approximately 1/1000 (Figure 1, Table 1). SAPP-injected mice had titers of 1:1,000 to 1:30,000 for this immunogen with only a single mice exceeding 1:10,0000.

The PBS-treated mice were titrated against aggregated  $A\beta_{42}$  at six, ten and twelve months. At a 1/100 dilution the PBS mice when titrated against aggregated  $A\beta_{42}$  only exceeded 4 times background at one data point, otherwise, they were less than 4 times background at all time points (Table 1). The SAP-specific response was negligible at these time points with all titers less than 300.

Seven out of the nine mice in the aggregated  $A\beta_{1-42}$  group had no detectable amyloid in their brains. In contrast, brain tissue from mice in the SAP and PBS groups contained numerous 3D6-positive amyloid deposits in the hippocampus, as well as in the frontal and cingulate cortices. The pattern of deposition was similar to that of untreated controls, with characteristic involvement of vulnerable subregions, such as the outer molecular layer of the hippocampal dentate gyrus. One mouse from the  $A\beta_{1-42}$ -injected group had a greatly reduced amyloid burden, confined to the hippocampus. An isolated plaque was identified in another  $A\beta_{1-42}$ -treated mouse.

Quantitative image analyses of the amyloid burden in the hippocampus verified the dramatic reduction achieved in the AN1792-treated animals (Fig. 2). The median values of the amyloid burden for the PBS group (2.22%), and for the untreated control group (2.65%) were significantly greater than for those immunized with AN1792 (0.00%,  $p=0.0005$ ). In contrast, the median value for the group immunized with SAP peptides (SAPP) was 5.74%. Brain tissue from the untreated,

control mice contained numerous A $\beta$  amyloid deposits visualized with the A $\beta$ -specific monoclonal antibody (mAb) 3D6 in the hippocampus, as well as in the retrosplenial cortex. A similar pattern of amyloid deposition was also seen in mice immunized with SAPP or PBS (Fig. 2). In addition, in these latter three groups there was a characteristic involvement of vulnerable subregions of the brain classically seen in AD, such as the outer molecular layer of the hippocampal dentate gyrus, in all three of these groups.

The brains that contained no A $\beta$  deposits were also devoid of neuritic plaques that are typically visualized in PDAPP mice with the human APP antibody 8E5. All of brains from the remaining groups (SAP-injected, PBS and uninjected mice) had numerous neuritic plaques typical of untreated PDAPP mice. A small number of neuritic plaques were present in one mouse treated with AN1792, and a single cluster of dystrophic neurites was found in a second mouse treated with AN1792. Image analyses of the hippocampus, and shown in Fig. 3, demonstrated the virtual elimination of dystrophic neurites in AN1792-treated mice (median 0.00%) compared to the PBS recipients (median 0.28%,  $p = 0.0005$ ).

Astrocytosis characteristic of plaque-associated inflammation was also absent in the brains of the A $\beta$ 1-42 injected group. The brains from the mice in the other groups contained abundant and clustered GFAP-positive astrocytes typical of A $\beta$  plaque-associated gliosis. A subset of the GFAP-reacted slides were counter-stained with Thioflavin S to localize the A $\beta$  deposits. The GFAP-positive astrocytes were associated with A $\beta$  plaques in the SAP, PBS and untreated controls. No such association was found in the plaque-negative A $\beta$ 1-42 treated mice, while minimal plaque-associated gliosis was identified in one mouse treated with AN1792.

Image analyses, shown in Fig. 4 for the retrosplenial cortex, verified that the reduction in astrocytosis was significant with a median value of 1.56% for those treated with AN1792 versus median values greater than 6% for groups immunized with SAP peptides, PBS or untreated ( $p=0.0017$ )

Evidence from a subset of the A $\beta$  1-42- and PBS-injected mice indicated plaque-associated MHC II immunoreactivity was absent in the A $\beta$ 1-42 injected mice, consistent with lack of an A $\beta$ -related inflammatory response.

5        Sections of the mouse brains were also reacted with a mA $\beta$  specific for MAC-1, a cell surface protein. MAC-1 (CD11b) is an integrin family member and exists as a heterodimer with CD18. The CD11b/CD18 complex is present on monocytes, macrophages, neutrophils and natural killer cells (Mak and  
10       Simard). The resident MAC-1-reactive cell type in the brain is likely to be microglia based on similar phenotypic morphology in MAC-1 immunoreacted sections. Plaque-associated MAC-1 labeling was lower in the brains of mice treated with AN1792 compared to the PBS control group, a finding consistent  
15       with the lack of an A $\beta$ -induced inflammatory response.

### C. Conclusion

The lack of A $\beta$  plaques and reactive neuronal and gliotic changes in the brains of the A $\beta$ 1-42-injected mice indicate that no or extremely little amyloid was deposited in their  
20       brains, and pathological consequences, such as gliosis and neuritic pathology, were absent. PDAPP mice treated with A $\beta$ 1-42 show essentially the same lack of pathology as control nontransgenic mice. Therefore, A $\beta$ 1-42 injections are highly effective in the prevention of deposition or clearance of  
25       human A $\beta$  from brain tissue, and elimination of subsequent neuronal and inflammatory degenerative changes. Thus, administration of A $\beta$  peptide has therapeutic benefit in prevention of AD.

### II. Dose Response Study

30       Groups of five-week old, female Swiss Webster mice (N=6 per group) were immunized with 300, 100, 33, 11, 3.7, 1.2, 0.4, or 0.13 ug of A $\beta$  formulated in CFA/IFA administered intraperitoneally. Three doses were given at biweekly intervals followed by a fourth dose one month later. The  
35       first dose was emulsified with CFA and the remaining doses were emulsified with IFA. Animals were bled 4-7 days

following each immunization starting after the second dose for measurement of antibody titers. Animals in a subset of three groups, those immunized with 11, 33, or 300  $\mu$ g of antigen, were additionally bled at approximately monthly intervals for four months following the fourth immunization to monitor the decay of the antibody response across a range of vaccine doses. These animals received a final fifth immunization at seven months after study initiation. They were sacrificed one week later to measure antibody responses to AN1792 and to perform toxicological analyses.

A declining dose response was observed from 300 to 3.7  $\mu$ g with no response at the two lowest doses. Mean antibody titers are about 1:1000 after 3 doses and about 1:10,000 after 4 doses of 11-300  $\mu$ g of antigen (see Fig. 5).

Antibody titers rose dramatically for all but the lowest dose group following the third immunization with increases in GMTs ranging from 5- to 25-fold. Low antibody responses were then detectable for even the 0.4  $\mu$ g recipients. The 1.2 and 3.7  $\mu$ g groups had comparable titers with GMTs of about 1000 and the highest four doses clustered together with GMTs of about 25,000, with the exception of the 33  $\mu$ g dose group with a lower GMT of 3000. Following the fourth immunization, the titer increase was more modest for most groups. There was a clear dose response across the lower antigen dose groups from 0.14  $\mu$ g to 11  $\mu$ g ranging from no detectable antibody for recipients of 0.14  $\mu$ g to a GMT of 36,000 for recipients of 11  $\mu$ g. Again, titers for the four highest dose groups of 11 to 300  $\mu$ g clustered together. Thus following two immunizations, the antibody titer was dependent on the antigen dose across the broad range from 0.4 to 300  $\mu$ g. By the third immunization, titers of the highest four doses were all comparable and they remained at a plateau after an additional immunization.

One month following the fourth immunization, titers were 2- to 3-fold higher in the 300  $\mu$ g group than those measured from blood drawn five days following the immunization (Fig. 6). This observation suggests that the peak anamnestic antibody response occurred later than 5 days post-immunization. A more

modest (50%) increase was seen at this time in the 33  $\mu$ g group. In the 300  $\mu$ g dose group at two months following the last dose, GMTs declined steeply by about 70%. After another month, the decline was less steep at 45% (100  $\mu$ g) and about 14% for the 33 and 11  $\mu$ g doses. Thus, the rate of decline in circulating antibody titers following cessation of immunization appears to be biphasic with a steep decline the first month following peak response followed by a more modest rate of decrease thereafter.

The antibody titers and the kinetics of the response of these Swiss Webster mice are similar to those of young heterozygous PDAPP transgenic mice immunized in a parallel manner. Dosages effective to induce an immune response in humans are typically similar to dosages effective in mice.

### III. Screen For Therapeutic Efficacy Against Established AD

This assay is designed to test immunogenic agents for activity in arresting or reversing neuropathological characteristics of AD in aged animals. Immunizations with 42 amino acid long A $\beta$  (AN1792) were begun at a timepoint when amyloid plaques are already present in the brains of the PDAPP mice.

Over the timecourse used in this study, untreated PDAPP mice develop a number of neurodegenerative changes that resemble those found in AD (Games et al., *supra* and Johnson-Wood et al., *Proc. Natl. Acad. Sci. USA* 94, 1550-1555 (1997)). The deposition of A $\beta$  into amyloid plaques is associated with a degenerative neuronal response consisting of aberrant axonal and dendritic elements, called dystrophic neurites. Amyloid deposits that are surrounded by and contain dystrophic neurites called neuritic plaques. In both AD and the PDAPP mouse, dystrophic neurites have a distinctive globular structure, are immunoreactive with a panel of antibodies recognizing APP and cytoskeletal components, and display complex subcellular degenerative changes at the ultrastructural level. These characteristics allow for disease-relevant, selective and reproducible measurements of neuritic plaque formation in the PDAPP brains. The dystrophic

neuronal component of PDAPP neuritic plaques is easily visualized with an antibody specific for human APP (mAb 8E5), and is readily measurable by computer-assisted image analysis. Therefore, in addition to measuring the effects of AN1792 on amyloid plaque formation, we monitored the effects of this treatment on the development of neuritic dystrophy.

Astrocytes and microglia are non-neuronal cells that respond to and reflect the degree of neuronal injury. GFAP-positive astrocytes and MHC II-positive microglia are commonly observed in AD, and their activation increases with the severity of the disease. Therefore, we also monitored the development of reactive astrocytosis and microgliosis in the AN1792-treated mice.

#### A. Materials and Methods

Forty-eight, heterozygous female PDAPP mice, 11 to 11.5 months of age, obtained from Charles River, were randomly divided into two groups: 24 mice to be immunized with 100  $\mu$ g of AN1792 and 24 mice to be immunized with PBS, each combined with Freund's adjuvant. The AN1792 and PBS groups were again divided when they reached ~15 months of age. At 15 months of age approximately half of each group of the AN1792- and PBS-treated animals were euthanized (n=10 and 9, respectively), the remainder continued to receive immunizations until termination at ~18 months (n=9 and 12, respectively). A total of 8 animals (5 AN1792, 3 PBS) died during the study. In addition to the immunized animals, one-year old (n=10), 15-month old (n=10) and 18-month old (n=10) untreated PDAPP mice were included for comparison in the ELISAs to measure a $\beta$  and APP levels in the brain; the one-year old animals were also included in the immunohistochemical analyses.

Methodology was as in Example 1 unless otherwise indicated. US Peptides lot 12 and California Peptides lot ME0339 of AN1792 were used to prepare the antigen for the six immunizations administered prior to the 15-month timepoint. California Peptides lots ME0339 and ME0439 were used for the three additional immunizations administered between 15 and 18 months.

For immunizations, 100  $\mu$ g of AN1792 in 200  $\mu$ l PBS or PBS alone was emulsified 1:1 (vol:vol) with Complete Freund's adjuvant (CFA) or Incomplete Freund's adjuvant (IFA) or PBS in a final volume of 400  $\mu$ l. The first immunization was delivered with CFA as adjuvant, the next four doses were given with IFA and the final four doses with PBS alone without added adjuvant. A total of nine immunizations were given over the seven-month period on a two-week schedule for the first three doses followed by a four-week interval for the remaining injections. The four-month treatment group, euthanized at 15 months of age, received only the first 6 immunizations.

## B. Results

### 1. Effects of AN1792 Treatment on Amyloid Burden

The results of AN1792 treatment on cortical amyloid burden determined by quantitative image analysis are shown in Fig. 7. The median value of cortical amyloid burden was 0.28% in a group of untreated 12-month old PDAPP mice, a value representative of the plaque load in mice at the study's initiation. At 18 months, the amyloid burden increased over 17-fold to 4.87% in PBS-treated mice, while AN1792-treated mice had a greatly reduced amyloid burden of only 0.01%, notably less than the 12-month untreated and both the 15- and 18-month PBS-treated groups. The amyloid burden was significantly reduced in the AN1792 recipients at both 15 (96% reduction;  $p=0.003$ ) and 18 (>99% reduction;  $p=0.0002$ ) months.

Typically, cortical amyloid deposition in PDAPP mice initiates in the frontal and retrosplenial cortices (RSC) and progresses in a ventral-lateral direction to involve the temporal and entorhinal cortices (EC). Little or no amyloid was found in the EC of 12 month-old mice, the approximate age at which AN1792 was first administered. After 4 months of AN1792 treatment, amyloid deposition was greatly diminished in the RSC, and the progressive involvement of the EC was entirely eliminated by AN1792 treatment. The latter observation showed that AN1792 completely halted the progression of amyloid that would normally invade the temporal

and ventral cortices, as well as arrested or possibly reversed deposition in the RSC.

The profound effects of AN1792 treatment on developing cortical amyloid burden in the PDAPP mice are further demonstrated by the 18-month group, which had been treated for seven months. A near complete absence of cortical amyloid was found in the AN1792-treated mouse, with a total lack of diffuse plaques, as well as a reduction in compacted deposits.

## 2. AN1792 Treatment-associated Cellular and Morphological Changes

A population of A $\beta$ -positive cells was found in brain regions that typically contain amyloid deposits. Remarkably, in several brains from AN1792 recipients, very few or no extracellular cortical amyloid plaques were found. Most of the A $\beta$  immunoreactivity appeared to be contained within cells with large lobular or clumped soma. Phenotypically, these cells resembled activated microglia or monocytes. They were immunoreactive with antibodies recognizing ligands expressed by activated monocytes and microglia (MHC II and CD11b) and were occasionally associated with the wall or lumen of blood vessels. Comparison of near-adjacent sections labeled with A $\beta$  and MHC II-specific antibodies revealed that similar patterns of these cells were recognized by both classes of antibodies. Detailed examination of the AN1792-treated brains revealed that the MHC II-positive cells were restricted to the vicinity of the limited amyloid remaining in these animals. Under the fixation conditions employed, the cells were not immunoreactive with antibodies that recognize T cell (CD3, CD3e) or B cell (CD45RA, CD45RB) ligands or leukocyte common antigen (CD45), but were reactive with an antibody recognizing leukosialin (CD43) which cross-reacts with monocytes. No such cells were found in any of the PBS-treated mice.

PDAPP mice invariably develop heavy amyloid deposition in the outer molecular layer of the hippocampal dentate gyrus. The deposition forms a distinct streak within the perforant pathway, a subregion that classically contains amyloid plaques in AD. The characteristic appearance of these deposits in PBS-treated mice resembled that previously characterized in



untreated PDAPP mice. The amyloid deposition consisted of both diffuse and compacted plaques in a continuous band. In contrast, in a number of brains from AN1792-treated mice this pattern was drastically altered. The hippocampal amyloid deposition no longer contained diffuse amyloid, and the banded pattern was completely disrupted. Instead, a number of unusual punctate structures were present that are reactive with anti-A $\beta$  antibodies, several of which appeared to be amyloid-containing cells.

MHC II-positive cells were frequently observed in the vicinity of extracellular amyloid in AN1792-treated animals. The pattern of association of A $\beta$ -positive cells with amyloid was very similar in several brains from AN1792-treated mice. The distribution of these monocytic cells was restricted to the proximity of the deposited amyloid and was entirely absent from other brain regions devoid of A $\beta$  plaques.

Quantitative image analysis of MHC II and MAC I-labeled sections revealed a trend towards increased immunoreactivity in the RSC and hippocampus of AN1792-treated mice compared to the PBS group which reached significance with the measure of MAC 1 reactivity in hippocampus.

These results are indicative of active, cell-mediated removal of amyloid in plaque-bearing brain regions.

### 3. AN1792 Effects on A $\beta$ Levels: ELISA Determinations

#### (a) Cortical Levels

In untreated PDAPP mice, the median level of total A $\beta$  in the cortex at 12 months was 1,600 ng/g, which increased to 8,700 ng/g by 15 months (Table 2). At 18 months the value was 22,000 ng/g, an increase of over 10-fold during the time course of the experiment. PBS-treated animals had 8,600 ng/g total A $\beta$  at 15 months which increased to 19,000 ng/g at 18 months. In contrast, AN1792-treated animals had 81% less total A $\beta$  at 15 months (1,600 ng/g) than the PBS-immunized group. Significantly less ( $p=0.0001$ ) total A $\beta$  (5,200 ng/g) was found at 18 months when the AN1792 and PBS groups were compared (Table 2), representing a 72% reduction in the A $\beta$  that would otherwise be present. Similar results were

obtained when cortical levels of A $\beta$ 42 were compared, namely that the AN1792-treated group contained much less A $\beta$ 42, but in this case the differences between the AN1792 and PBS groups were significant at both 15 months ( $p=0.04$ ) and 18 months ( $p=0.0001$ , Table 2).

**Table 2: Median A $\beta$  Levels (ng/g) in Cortex**

| Age | UNTREATED       |              |      | PBS             |              |      | AN1792  |              |      |
|-----|-----------------|--------------|------|-----------------|--------------|------|---------|--------------|------|
|     | Total A $\beta$ | A $\beta$ 42 | (n)  | Total A $\beta$ | A $\beta$ 42 | (n)  | Total   | A $\beta$ 42 | (n)  |
| 12  | 1,600           | 1,300        | (10) |                 |              |      |         |              |      |
| 15  | 8,700           | 8,300        | (10) | 8,600           | 7,200        | (9)  | 1,600   | 1,300*       | (10) |
| 18  | 22,200          | 18,500       | (10) | 19,000          | 15,900       | (12) | 5,200** | 4,000**      | (9)  |

\*  $p = 0.0412$

\*\*  $p = 0.0001$

(b) Hippocampal Levels

In untreated PDAPP mice, median hippocampal levels of total A $\beta$  at twelve months of age were 15,000 ng/g which increased to 51,000 ng/g at 15 months and further to 81,000 ng/g at 18 months (Table 3). Similarly, PBS immunized mice showed values of 40,000 ng/g and 65,000 ng/g at 15 months and 18 months, respectively. AN1792 immunized animals exhibited less total A $\beta$ , specifically 25,000 ng/g and 51,000 ng/g at the respective 15-month and 18-month timepoints. The 18-month AN1792-treated group value was significantly lower than that of the PBS treated group ( $p= 0.0105$ ; Table 3). Measurement of A $\beta$ 42 gave the same pattern of results, namely that levels in the AN1792-treated group were significantly lower than in the PBS group (39,000 ng/g vs. 57,000 ng/g, respectively;  $p=0.0022$ ) at the 18-month evaluation (Table 3).

**Table 3: Median A $\beta$  Levels (ng/g) in Hippocampus**

| Age | UNTREATED       |              |      | PBS             |              |      | AN1792  |              |      |
|-----|-----------------|--------------|------|-----------------|--------------|------|---------|--------------|------|
|     | Total A $\beta$ | A $\beta$ 42 | (n)  | Total A $\beta$ | A $\beta$ 42 | (n)  | Total   | A $\beta$ 42 | (n)  |
| 12  | 15,500          | 11,100       | (10) |                 |              |      |         |              |      |
| 15  | 51,500          | 44,400       | (10) | 40,100          | 35,700       | (9)  | 24,500  | 22,100       | (10) |
| 18  | 80,800          | 64,200       | (10) | 65,400          | 57,100       | (12) | 50,900* | 38,900**     | (9)  |

\* p = 0.0105

\*\* p = 0.0022

**(c) Cerebellar Levels**

In 12-month untreated PDAPP mice, the median cerebellar level of total A $\beta$  was 15 ng/g (Table 4). At 15 months, this median increased to 28 ng/g and by 18 months had risen to 35 ng/g. PBS-treated animals displayed median total A $\beta$  values of 21 ng/g at 15 months and 43 ng/g at 18 months. AN1792-treated animals were found to have 22 ng/g total A $\beta$  at 15 months and significantly less (p=0.002) total A $\beta$  at 18 months (25 ng/g) than the corresponding PBS group (Table 4).

**Table 4: Median A $\beta$  Levels (ng/g) in Cerebellum**

| Age (months) | UNTREATED       |      | PBS             |      | AN1792          |      |
|--------------|-----------------|------|-----------------|------|-----------------|------|
|              | Total A $\beta$ | (n)  | Total A $\beta$ | (n)  | Total A $\beta$ | (n)  |
| 12           | 15.6            | (10) |                 |      |                 |      |
| 15           | 27.7            | (10) | 20.8            | (9)  | 21.7            | (10) |
| 18           | 35.0            | (10) | 43.1            | (12) | 24.8*           | (9)  |

\* p = 0.0018

**4. Effects of AN1792 Treatment on APP Levels**

APP- $\alpha$  and the full-length APP molecule both contain all or part of the A $\beta$  sequence and thus could be potentially impacted by the generation of an AN1792-directed immune response. In studies to date, a slight increase in APP levels has been noted as neuropathology increases in the PDAPP mouse. In the cortex, levels of either APP- $\alpha$ /FL (full length) or APP- $\alpha$  were essentially unchanged by treatment with the exception that APP- $\alpha$  was reduced by 19% at the 18-month timepoint in the AN1792-treated vs. the PBS-treated group. The 18-month AN1792-treated APP values were not significantly different

from values of the 12-month and 15-month untreated and 15-month PBS groups. In all cases the APP values remained within the ranges that are normally found in PDAPP mice.

#### 5. Effects of AN1792 Treatment on Neurodegenerative and Gliotic Pathology

Neuritic plaque burden was significantly reduced in the frontal cortex of AN1792-treated mice compared to the PBS group at both 15 (84%;  $p=0.03$ ) and 18 (55%;  $p=0.01$ ) months of age (Fig. 8). The median value of the neuritic plaque burden increased from 0.32% to 0.49% in the PBS group between 15 and 18 months of age. This contrasted with the greatly reduced development of neuritic plaques in the AN1792 group, with median neuritic plaque burden values of 0.05% and 0.22%, in the 15 and 18 month groups, respectively.

Immunizations with AN1792 seemed well tolerated and reactive astrogliosis was also significantly reduced in the RSC of AN1792-treated mice when compared to the PBS group at both 15 (56%;  $p=0.011$ ) and 18 (39%;  $p=0.028$ ) months of age (Fig. 9). Median values of the percent of astrogliosis in the PBS group increased between 15 and 18 months from 4.26% to 5.21%. AN1792-treatment suppressed the development of astrogliosis at both time points to 1.89% and 3.2%, respectively. This suggests the neuropil was not being damaged by the clearance process.

#### 6. Antibody Responses

As described above, eleven-month old, heterozygous PDAPP mice ( $N=24$ ) received a series of 5 immunizations of 100  $\mu$ g of AN1792 emulsified with Freund's adjuvant and administered intraperitoneally at weeks 0, 2, 4, 8, and 12, and a sixth immunization with PBS alone (no Freund's adjuvant) at week 16. As a negative control, a parallel set of 24 age-matched transgenic mice received immunizations of PBS emulsified with the same adjuvants and delivered on the same schedule. Animals were bled within three to seven days following each immunization starting after the second dose. Antibody responses to AN1792 were measured by ELISA. Geometric mean titers (GMT) for the animals that were immunized with AN1792

were approximately 1,900, 7,600, and 45,000 following the second, third and last (sixth) doses respectively. No A $\beta$ -specific antibody was measured in control animals following the sixth immunization.

5        Approximately one-half of the animals were treated for an additional three months, receiving immunizations at about 20, 24 and 27 weeks. Each of these doses was delivered in PBS vehicle alone without Freund's adjuvant. Mean antibody titers remained unchanged over this time period. In fact, antibody  
10        titers appeared to remain stable from the fourth to the eighth bleed corresponding to a period covering the fifth to the ninth injections.

      To determine if the A $\beta$ -specific antibodies elicited by immunization that were detected in the sera of AN1792-treated  
15        mice were also associated with deposited brain amyloid, a subset of sections from the AN1792- and PBS-treated mice were reacted with an antibody specific for mouse IgG. In contrast to the PBS group, A $\beta$  plaques in AN1792-treated brains were coated with endogenous IgG. This difference between the two  
20        groups was seen in both 15- and 18-month groups. Particularly striking was the lack of labeling in the PBS group, despite the presence of a heavy amyloid burden in these mice. These results show that immunization with a synthetic A $\beta$  protein generates antibodies that recognize and bind *in vivo* to the A $\beta$   
25        in amyloid plaques.

#### 7. Cellular-Mediated Immune Responses

      Spleens were removed from nine AN1792-immunized and 12 PBS-immunized 18-month old PDAPP mice 7 days after the ninth immunization. Splenocytes were isolated and cultured for 72 h  
30        in the presence of A $\beta$ 40, A $\beta$ 42, or A $\beta$ 40-1 (reverse order protein). The mitogen Con A served as a positive control. Optimum responses were obtained with >1.7  $\mu$ M protein. Cells from all nine AN1792-treated animals proliferated in response to either A $\beta$ 1-40 or A $\beta$ 1-42 protein, with equal levels of  
35        incorporation for both proteins (Fig. 10, Upper Panel). There was no response to the A $\beta$ 40-1 reverse protein. Cells from

control animals did not respond to any of the A $\beta$  proteins (Fig. 10, Lower Panel).

### C. Conclusion

The results of this study show that AN1792 immunization of PDAPP mice possessing existing amyloid deposits slows and prevents progressive amyloid deposition and retard consequential neuropathological changes in the aged PDAPP mouse brain. Immunizations with AN1792 essentially halted amyloid developing in structures that would normally succumb to amyloidosis. Thus, administration of A $\beta$  peptide has therapeutic benefit in the treatment of AD.

### IV. Screen of A $\beta$ Fragments

100 PDAPP mice age 9-11 months are immunized with 9 different regions of APP and A $\beta$  to determine which epitopes convey the response. The 9 different immunogens and one control are injected i.p. as described above. The immunogens include four human A $\beta$  peptide conjugates 1-12, 13-28, 32-42, 1-5, all coupled to sheep anti-mouse IgG via a cystine link; an APP polypeptide aa 592-695, aggregated human A $\beta$  1-40, and aggregated human A $\beta$  25-35, and aggregated rodent A $\beta$ 42. Aggregated A $\beta$ 42 and PBS are used as controls. Ten mice are used per treatment group. Titers are monitored as above and mice are euthanized at the end of 4 months of injections. Histochemistry, A $\beta$  levels, and toxicology are determined post mortem.

### A. Materials and Methods

#### 1. Preparation of Immunogens

Preparation of coupled A $\beta$  peptides: four human A $\beta$  peptide conjugates (amino acid residues 1-5, 1-12, 13-28, and 33-42, each conjugated to sheep anti-mouse IgG) were prepared by coupling through an artificial cysteine added to the A $\beta$  peptide using the crosslinking reagent sulfo-EMCS. The A $\beta$  peptide derivatives were synthesized with the following final amino acid sequences. In each case, the location of the inserted cysteine residue is indicated by underlining. The

A $\beta$ 13-28 peptide derivative also had two glycine residues added prior to the carboxyl terminal cysteine as indicated.

A $\beta$ 1-12 peptide     NH<sub>2</sub>-DAEFRHDSGYEVC COOH

A $\beta$ 1-5 peptide     NH<sub>2</sub>-DAEFRC COOH

5     A $\beta$ 33-42 peptide     NH<sub>2</sub>-C-amino-heptanoic acid-GLMVGGVVIA COOH

A $\beta$ 13-28 peptide     Ac-NH-HHQLVFFAEDVGSNKGGC-COOH

To prepare for the coupling reaction, ten mg of sheep anti-mouse IgG (Jackson ImmunoResearch Laboratories) was dialyzed overnight against 10 mM sodium borate buffer, pH 8.5.

10     The dialyzed antibody was then concentrated to a volume of 2 mL using an Amicon Centriprep tube. Ten mg sulfo-EMCS [N ( $\epsilon$ -maleimidocaproyloxy) succinimide] (Molecular Sciences Co.) was dissolved in one mL deionized water. A 40-fold molar excess of sulfo-EMCS was added dropwise with stirring to the

15     sheep anti-mouse IgG and then the solution was stirred for an additional ten min. The activated sheep anti-mouse IgG was purified and buffer exchanged by passage over a 10 mL gel filtration column (Pierce Presto Column, obtained from Pierce Chemicals) equilibrated with 0.1 M NaPO<sub>4</sub>, 5 mM EDTA, pH 6.5.

20     Antibody containing fractions, identified by absorbance at 280 nm, were pooled and diluted to a concentration of approximately 1 mg/mL, using 1.4 mg per OD as the extinction coefficient. A 40-fold molar excess of A $\beta$  peptide was dissolved in 20 mL of 10 mM NaPO<sub>4</sub>, pH 8.0, with the exception

25     of the A $\beta$ 33-42 peptide for which 10 mg was first dissolved in 0.5 mL of DMSO and then diluted to 20 mL with the 10 mM NaPO<sub>4</sub> buffer. The peptide solutions were each added to 10 mL of activated sheep anti-mouse IgG and rocked at room temperature for 4 hr. The resulting conjugates were concentrated to a

30     final volume of less than 10 mL using an Amicon Centriprep tube and then dialyzed against PBS to buffer exchange the buffer and remove free peptide. The conjugates were passed through 0.22  $\mu$ -pore size filters for sterilization and then aliquoted into fractions of 1 mg and stored frozen at -20°C.

35     The concentrations of the conjugates were determined using the BCA protein assay (Pierce Chemicals) with horse IgG for the

standard curve. Conjugation was documented by the molecular weight increase of the conjugated peptides relative to that of the activated sheep anti-mouse IgG. The A $\beta$  1-5 sheep anti-mouse conjugate was a pool of two conjugations, the rest were from a single preparation.

## 2. Preparation of aggregated A $\beta$ peptides

Human 1-40 (AN1528; California Peptides Inc., Lot ME0541), human 1-42 (AN1792; California Peptides Inc., Lots ME0339 and ME0439), human 25-35, and rodent 1-42 (California Peptides Inc., Lot ME0218) peptides were freshly solubilized for the preparation of each set of injections from lyophilized powders that had been stored desiccated at -20°C. For this purpose, two mg of peptide were added to 0.9 ml of deionized water and the mixture was vortexed to generate a relatively uniform solution or suspension. Of the four, AN1528 was the only peptide soluble at this step. A 100  $\mu$ l aliquot of 10X PBS (1X PBS: 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5) was then added at which point AN1528 began to precipitate. The suspension was vortexed again and incubated overnight at 37°C for use the next day.

Preparation of the pBx6 protein: An expression plasmid encoding pBx6, a fusion protein consisting of the 100-amino acid bacteriophage MS-2 polymerase N-terminal leader sequence followed by amino acids 592-695 of APP ( $\beta$ APP) was constructed as described by Oltersdorf et al., *J. Biol. Chem.* 265, 4492-4497 (1990). The plasmid was transfected into *E. coli* and the protein was expressed after induction of the promoter. The bacteria were lysed in 8M urea and pBx6 was partially purified by preparative SDS PAGE. Fractions containing pBx6 were identified by Western blot using a rabbit anti-pBx6 polyclonal antibody, pooled, concentrated using an Amicon Centriprep tube and dialysed against PBS. The purity of the preparation, estimated by Coomassie Blue stained SDS PAGE, was approximately 5 to 10%.



## B. Results and Discussion

### 1. Study Design

One hundred male and female, nine- to eleven-month old heterozygous PDAPP transgenic mice were obtained from Charles River Laboratory and Taconic Laboratory. The mice were sorted into ten groups to be immunized with different regions of A $\beta$  or APP combined with Freund's adjuvant. Animals were distributed to match the gender, age, parentage and source of the animals within the groups as closely as possible. The immunogens included four A $\beta$  peptides derived from the human sequence, 1-5, 1-12, 13-28, and 33-42, each conjugated to sheep anti-mouse IgG; four aggregated A $\beta$  peptides, human 1-40 (AN1528), human 1-42 (AN1792), human 25-35, and rodent 1-42; and a fusion polypeptide, designated as pBx6, containing APP amino acid residues 592-695. A tenth group was immunized with PBS combined with adjuvant as a control.

For each immunization, 100  $\mu$ g of each A $\beta$  peptide in 200  $\mu$ l PBS or 200  $\mu$ g of the APP derivative pBx6 in the same volume of PBS or PBS alone was emulsified 1:1 (vol:vol) with Complete Freund's adjuvant (CFA) in a final volume of 400  $\mu$ l for the first immunization, followed by a boost of the same amount of immunogen in Incomplete Freund's adjuvant (IFA) for the subsequent four doses and with PBS for the final dose. Immunizations were delivered intraperitoneally on a biweekly schedule for the first three doses, then on a monthly schedule thereafter. Animals were bled four to seven days following each immunization starting after the second dose for the measurement of antibody titers. Animals were euthanized approximately one week after the final dose.

### 2. A $\beta$ and APP Levels in the Brain

Following about four months of immunization with the various A $\beta$  peptides or the APP derivative, brains were removed from saline-perfused animals. One hemisphere was prepared for immunohistochemical analysis and the second was used for the quantitation of A $\beta$  and APP levels. To measure the concentrations of various forms of beta amyloid peptide and amyloid precursor protein, the hemisphere was dissected and

homogenates of the hippocampal, cortical, and cerebellar regions were prepared in 5 M guanidine. These were diluted and the level of amyloid or APP was quantitated by comparison to a series of dilutions of standards of A $\beta$  peptide or APP of known concentrations in an ELISA format.

The median concentration of total A $\beta$  for the control group immunized with PBS was 5.8-fold higher in the hippocampus than in the cortex (median of 24,318 ng/g hippocampal tissue compared to 4,221 ng/g for the cortex). The median level in the cerebellum of the control group (23.4 ng/g tissue) was about 1,000-fold lower than in the hippocampus. These levels are similar to those that we have previously reported for heterozygous PDAPP transgenic mice of this age (Johnson-Woods et al., 1997, supra).

For the cortex, a subset of treatment groups had median total A $\beta$  and A $\beta$ 1-42 levels which differed significantly from those of the control group ( $p < 0.05$ ), those animals receiving AN1792, rodent A $\beta$ 1-42 or the A $\beta$ 1-5 peptide conjugate as shown in **Fig. 11**. The median levels of total A $\beta$  were reduced by 75%, 79% and 61%, respectively, compared to the control for these treatment groups. There were no discernable correlations between A $\beta$ -specific antibody titers and A $\beta$  levels in the cortical region of the brain for any of the groups.

In the hippocampus, the median reduction of total A $\beta$  associated with AN1792 treatment (46%,  $p = 0.0543$ ) was not as great as that observed in the cortex (75%,  $p = 0.0021$ ). However, the magnitude of the reduction was far greater in the hippocampus than in the cortex, a net reduction of 11,186 ng/g tissue in the hippocampus versus 3,171 ng/g tissue in the cortex. For groups of animals receiving rodent A $\beta$ 1-42 or A $\beta$ 1-5, the median total A $\beta$  levels were reduced by 36% and 26%, respectively. However, given the small group sizes and the high variability of the amyloid peptide levels from animal to animal within both groups, these reductions were not significant. When the levels of A $\beta$ 1-42 were measured in the hippocampus, none of the treatment-induced reductions reached significance. Thus, due to the smaller A $\beta$  burden in the cortex, changes in this region are a more sensitive indicator

of treatment effects. The changes in A $\beta$  levels measured by ELISA in the cortex are similar, but not identical, to the results from the immunohistochemical analysis (see below).

Total A $\beta$  was also measured in the cerebellum, a region typically unaffected in the AD pathology. None of the median A $\beta$  concentrations of any of the groups immunized with the various A $\beta$  peptides or the APP derivative differed from that of the control group in this region of the brain. This result suggests that non-pathological levels of A $\beta$  are unaffected by treatment.

APP concentration was also determined by ELISA in the cortex and cerebellum from treated and control mice. Two different APP assays were utilized. The first, designated APP- $\alpha$ /FL, recognizes both APP-alpha ( $\alpha$ , the secreted form of APP which has been cleaved within the A $\beta$  sequence), and full-length forms (FL) of APP, while the second recognizes only APP- $\alpha$ . In contrast to the treatment-associated diminution of A $\beta$  in a subset of treatment groups, the levels of APP were unchanged in all of the treated compared to the control animals. These results indicate that the immunizations with A $\beta$  peptides are not depleting APP; rather the treatment effect is specific to A $\beta$ .

In summary, total A $\beta$  and A $\beta$ 1-42 levels were significantly reduced in the cortex by treatment with AN1792, rodent A $\beta$ 1-42 or A $\beta$ 1-5 conjugate. In the hippocampus, total A $\beta$  was significantly reduced only by AN1792 treatment. No other treatment-associated changes in A $\beta$  or APP levels in the hippocampal, cortical or cerebellar regions were significant.

## 2. Histochemical Analyses

Brains from a subset of six groups were prepared for immunohistochemical analysis, three groups immunized with the A $\beta$  peptide conjugates A $\beta$ 1-5, A $\beta$ 1-12, and A $\beta$ 13-28; two groups immunized with the full length A $\beta$  aggregates AN1792 and AN1528 and the PBS-treated control group. The results of image analyses of the amyloid burden in brain sections from these groups are shown in Fig. 12. There were significant reductions of amyloid burden in the cortical regions of three

of the treatment groups versus control animals. The greatest reduction of amyloid burden was observed in the group receiving AN1792 where the mean value was reduced by 97% ( $p = 0.001$ ). Significant reductions were also observed for those animals treated with AN1528 (95%,  $p = 0.005$ ) and the A $\beta$ 1-5 peptide conjugate (67%,  $p = 0.02$ ).

The results obtained by quantitation of total A $\beta$  or A $\beta$ 1-42 by ELISA and amyloid burden by image analysis differ to some extent. Treatment with AN1528 had a significant impact on the level of cortical amyloid burden when measured by quantitative image analysis but not on the concentration of total A $\beta$  in the same region when measured by ELISA. The difference between these two results is likely to be due to the specificities of the assays. Image analysis measures only insoluble A $\beta$  aggregated into plaques. In contrast, the ELISA measures all forms of A $\beta$ , both soluble and insoluble, monomeric and aggregated. Since the disease pathology is thought to be associated with the insoluble plaque-associated form of A $\beta$ , the image analysis technique may have more sensitivity to reveal treatment effects. However since the ELISA is a more rapid and easier assay, it is very useful for screening purposes. Moreover it may reveal that the treatment-associated reduction of A $\beta$  is greater for plaque-associated than total A $\beta$ .

To determine if the A $\beta$ -specific antibodies elicited by immunization in the treated animals reacted with deposited brain amyloid, a subset of the sections from the treated animals and the control mice were reacted with an antibody specific for mouse IgG. In contrast to the PBS group, A $\beta$ -containing plaques were coated with endogenous IgG for animals immunized with the A $\beta$  peptide conjugates A $\beta$ 1-5, A $\beta$ 1-12, and A $\beta$ 13-28; and the full length A $\beta$  aggregates AN1792 and AN1528. Brains from animals immunized with the other A $\beta$  peptides or the APP peptide pBx6 were not analyzed by this assay.

### 3. Measurement of Antibody Titers

Mice were bled four to seven days following each immunization starting after the second immunization, for a

total of five bleeds. Antibody titers were measured as A $\beta$ 1-42-binding antibody using a sandwich ELISA with plastic multi-well plates coated with A $\beta$ 1-42. As shown in Fig. 13, peak antibody titers were elicited following the fourth dose for those four vaccines which elicited the highest titers of AN1792-specific antibodies: AN1792 (peak GMT: 94,647), AN1528 (peak GMT: 88,231), A $\beta$ 1-12 conjugate (peak GMT: 47,216) and rodent A $\beta$ 1-42 (peak GMT: 10,766). Titers for these groups declined somewhat following the fifth and sixth doses. For the remaining five immunogens, peak titers were reached following the fifth or the sixth dose and these were of much lower magnitude than those of the four highest titer groups: A $\beta$ 1-5 conjugate (peak GMT: 2,356), pBx6 (peak GMT: 1,986), A $\beta$ 13-28 conjugate (peak GMT: 1,183), A $\beta$ 33-42 conjugate (peak GMT: 658), A $\beta$ 25-35 (peak GMT: 125). Antibody titers were also measured against the homologous peptides using the same ELISA sandwich format for a subset of the immunogens, those groups immunized with A $\beta$ 1-5, A $\beta$ 13-28, A $\beta$ 25-35, A $\beta$ 33-42 or rodent A $\beta$ 1-42. These titers were about the same as those measured against A $\beta$ 1-42 except for the rodent A $\beta$ 1-42 immunogen in which case antibody titers against the homologous immunogen were about two-fold higher. The magnitude of the AN1792-specific antibody titer of individual animals or the mean values of treatment groups did not correlate with efficacy measured as the reduction of A $\beta$  in the cortex.

#### 4. Lymphoproliferative Responses

A $\beta$ -dependent lymphoproliferation was measured using spleen cells harvested approximately one week following the final, sixth, immunization. Freshly harvested cells,  $10^5$  per well, were cultured for 5 days in the presence of A $\beta$ 1-40 at a concentration of 5  $\mu$ M for stimulation. Cells from a subset of seven of the ten groups were also cultured in the presence of the reverse peptide, A $\beta$ 40-1. As a positive control, additional cells were cultured with the T cell mitogen, PHA, and, as a negative control, cells were cultured without added peptide.

Lymphocytes from a majority of the animals proliferated in response to PHA. There were no significant responses to the A $\beta$ 40-1 reverse peptide. Cells from animals immunized with the larger aggregated A $\beta$  peptides, AN1792, rodent A $\beta$ 1-42 and AN1528 proliferated robustly when stimulated with A $\beta$ 1-40 with the highest cpm in the recipients of AN1792. One animal in each of the groups immunized with A $\beta$ 1-12 conjugate, A $\beta$ 13-28 conjugate and A $\beta$ 25-35 proliferated in response to A $\beta$ 1-40. The remaining groups receiving A $\beta$ 1-5 conjugate, A $\beta$ 33-42 conjugate pBx6 or PBS had no animals with an A $\beta$ -stimulated response. These results are summarized in Table 5 below.

| Table 5          |           |                       |            |
|------------------|-----------|-----------------------|------------|
| Immunogen        | Conjugate | A $\beta$ Amino Acids | Responders |
| A $\beta$ 1-5    | yes       | 5-mer                 | 0/7        |
| A $\beta$ 1-12   | yes       | 12-mer                | 1/8        |
| A $\beta$ 13-28  | yes       | 16-mer                | 1/9        |
| A $\beta$ 25-35  |           | 11-mer                | 1/9        |
| A $\beta$ 33-42  | yes       | 11-mer                | 0/10       |
| A $\beta$ 1-40   |           | 40-mer                | 0/8        |
| A $\beta$ 1-42   |           | 42-mer                | 9/9        |
| r A $\beta$ 1-42 |           | 42-mer                | 8/8        |
| pBx6             |           |                       | 0/8        |
| PBS              |           | 0-mer                 | 0/8        |

These results show that AN1792 and AN1528 stimulate strong T cell responses, most likely of the CD4<sup>+</sup> phenotype. The absence of an A $\beta$ -specific T cell response in animals immunized with A $\beta$ 1-5 is not surprising since peptide epitopes recognized by CD4<sup>+</sup> T cells are usually about 15 amino acids in length, although shorter peptides can sometimes function with less efficiency. Thus the majority of helper T cell epitopes for the four conjugate peptides are likely to reside in the IgG conjugate partner, not in the A $\beta$  region. This hypothesis is supported by the very low incidence of proliferative responses for animals in each of these treatment groups. Since the A $\beta$ 1-5 conjugate was effective at significantly reducing the level

of A $\beta$  in the brain, in the apparent absence of A $\beta$ -specific T cells, the key effector immune response induced by immunization with this peptide appears to be antibody.

Lack of T-cell and low antibody response from fusion peptide pBx6, encompassing APP amino acids 592-695 including all of the A $\beta$  residues may be due to the poor immunogenicity of this particular preparation. The poor immunogenicity of the A $\beta$ 25-35 aggregate is likely due to the peptide being too small to be likely to contain a good T cell epitope to help the induction of an antibody response. If this peptide were conjugated to a carrier protein, it would probably be more immunogenic.

#### V. Preparation of Polyclonal Antibodies for Passive Protection

20 non-transgenic mice are immunized with A $\beta$  or other immunogen, optionally plus adjuvant, and are euthanized at 4-5 months. Blood is collected from immunized mice. Optionally, IgG is separated from other blood components. Antibody specific for the immunogen may be partially purified by affinity chromatography. An average of about 0.5-1 mg of immunogen-specific antibody is obtained per mouse, giving a total of 5-10 mg.

#### VI. Passive Immunization with Antibodies to A $\beta$

Groups of 7-9 month old PDAPP mice each are injected with 0.5 mg in PBS of polyclonal anti-A $\beta$  or specific anti-A $\beta$  monoclonals as shown below. All antibody preparations are purified to have low endotoxin levels. Monoclonals can be prepared against a fragment by injecting the fragment or longer form of A $\beta$  into a mouse, preparing hybridomas and screening the hybridomas for an antibody that specifically

binds to a desired fragment of A $\beta$  without binding to other nonoverlapping fragments of A $\beta$ .

Table 6

| Antibody                                    | Epitope                      |
|---------------------------------------------|------------------------------|
| 2H3                                         | A $\beta$ 1-12               |
| 10D5                                        | A $\beta$ 1-12               |
| 266                                         | A $\beta$ 13-28              |
| 21F12                                       | A $\beta$ 33-42              |
| Mouse polyclonal<br>anti-human A $\beta$ 42 | Anti-Aggregated A $\beta$ 42 |

Mice are injected ip as needed over a 4 month period to maintain a circulating antibody concentration measured by ELISA titer of greater than 1/1000 defined by ELISA to A $\beta$ 42 or other immunogen. Titers are monitored as above and mice are euthanized at the end of 4 months of injections. Histochemistry, A $\beta$  levels and toxicology are performed post mortem. Ten mice are used per group.

#### VII. Comparison of Different Adjuvants

This examples compares CFA, alum, an oil-in water emulsion and MPL for capacity to stimulate an immune response.



## A. Materials and Methods

### 1. Study Design

One hundred female Hartley strain six-week old guinea pigs, obtained from Elm Hill, were sorted into ten groups to be immunized with AN1792 or a palmitoylated derivative thereof combined with various adjuvants. Seven groups received injections of AN1792 (33 $\mu$ g unless otherwise specified) combined with a) PBS, b) Freund's adjuvant, c) MPL, d) squalene, e) MPL/squalene f) low dose alum, or g) high dose alum (300 $\mu$ g AN1792). Two groups received injections of a palmitoylated derivative of AN1792 (33  $\mu$ g) combined with a) PBS or b) squalene. A final, tenth group received PBS alone without antigen or additional adjuvant. For the group receiving Freund's adjuvant, the first dose was emulsified with CFA and the remaining four doses with IFA. Antigen was administered at a dose of 33  $\mu$ g for all groups except the high dose alum group, which received 300  $\mu$ g of AN1792. Injections were administered intraperitoneally for CFA/IFA and intramuscularly in the hind limb quadriceps alternately on the right and left side for all other groups. The first three doses were given on a biweekly schedule followed by two doses at a monthly interval). Blood was drawn six to seven days following each immunization, starting after the second dose, for measurement of antibody titers.

### 2. Preparation of Immunogens

Two mg A $\beta$ 42 (California Peptide, Lot ME0339) was added to 0.9 ml of deionized water and the mixture was vortexed to generate a relatively uniform suspension. A 100  $\mu$ l aliquot of 10X PBS (1X PBS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5) was added. The suspension was vortexed again and incubated overnight at 37°C for use the next day. Unused A $\beta$ 1-42 was stored with desiccant as a lyophilized powder at -20°C.

A palmitoylated derivative of AN1792 was prepared by coupling palmitic anhydride, dissolved in dimethyl formamide, to the amino terminal residue of AN1792 prior to removal of the nascent peptide from the resin by treatment with hydrofluoric acid.

To prepare vaccine doses with Complete Freund's adjuvant (CFA) (group 2), 33  $\mu$ g of AN1792 in 200  $\mu$ l PBS was emulsified 1:1 (vol:vol) with CFA in a final volume of 400  $\mu$ l for the first immunization. For subsequent immunizations, the antigen was similarly emulsified with Incomplete Freund's adjuvant (IFA).

To prepare vaccine doses with MPL for groups 5 and 8, lyophilized powder (Ribi ImmunoChem Research, Inc., Hamilton, MT) was added to 0.2% aqueous triethylamine to a final concentration of 1 mg/ml and vortexed. The mixture was heated to 65 to 70°C for 30 sec to create a slightly opaque uniform suspension of micelles. The solution was freshly prepared for each set of injections. For each injection in group 5, 33  $\mu$ g of AN1792 in 16.5  $\mu$ l PBS, 50  $\mu$ g of MPL (50  $\mu$ l) and 162  $\mu$ l of PBS were mixed in a borosilicate tube immediately before use.

To prepare vaccine doses with the low oil-in-water emulsion, AN1792 in PBS was added to 5% squalene, 0.5% Tween 80, 0.5% Span 85 in PBS to reach a final single dose concentration of 33  $\mu$ g AN1792 in 250  $\mu$ l (group 6). The mixture was emulsified by passing through a two-chambered hand-held device 15 to 20 times until the emulsion droplets appeared to be about equal in diameter to a 1.0  $\mu$ m diameter standard latex bead when viewed under a microscope. The resulting suspension was opalescent, milky white. The emulsions were freshly prepared for each series of injections. For group 8, MPL in 0.2% triethylamine was added at a concentration of 50  $\mu$ g per dose to the squalene and detergent mixture for emulsification as noted above. For the palmitoyl derivative (group 7), 33  $\mu$ g per dose of palmitoyl-NH-A $\beta$ 1-42 was added to squalene and vortexed. Tween 80 and Span 85 were then added with

vortexing. This mixture was added to PBS to reach final concentrations of 5% squalene, 0.5% Tween 80, 0.5% Span 85 and the mixture was emulsified as noted above.

To prepare vaccine doses with alum (groups 9 and 10), AN1792 in PBS was added to Alhydrogel (aluminum hydroxide gel, Accurate, Westbury, NY) to reach concentrations of 33  $\mu\text{g}$  (low dose, group 9) or 300  $\mu\text{g}$  (high dose, group 10) AN1792 per 5 mg of alum in a final dose volume of 250  $\mu\text{l}$ . The suspension was gently mixed for 4 hr at RT.

### 3. Measurement of Antibody Titers

Guinea pigs were bled six to seven days following immunization starting after the second immunization for a total of four bleeds. Antibody titers against A $\beta$ 42 were measured by ELISA as described in General Materials and Methods.

### 4. Tissue Preparation

. After about 14 weeks, all guinea pigs were administered CO<sub>2</sub>. Cerebrospinal fluid was collected and the brains were removed and three brain regions (hippocampus, cortex and cerebellum) were dissected and used to measure the concentration of total A $\beta$  protein using ELISA.

## B. Results

### 1. Antibody Responses

There was a wide range in the potency of the various adjuvants when measured as the antibody response to AN1792 following immunization. As shown in **Fig. 14**, when AN1792 was administered in PBS, no antibody was detected following two or three immunizations and negligible responses were detected following the fourth and fifth doses with geometric mean titers (GMTs) of only about 45. The o/w emulsion induced modest titers following the third dose (GMT 255) that were maintained following the fourth dose (GMT 301) and fell with the final dose (GMT 54). There was a clear antigen dose response for AN1792 bound to alum with 300  $\mu$ g being more immunogenic at all time points than 33  $\mu$ g. At the peak of the antibody response, following the fourth immunization, the difference between the two doses was 43% with GMTs of about 1940 (33  $\mu$ g) and 3400 (300  $\mu$ g). The antibody response to 33  $\mu$ g AN1792 plus MPL was very similar to that generated with almost a ten-fold higher dose of antigen (300  $\mu$ g) bound to alum. The addition of MPL to an o/w emulsion decreased the potency of the vaccine relative to that with MPL as the sole adjuvant by as much as 75%. A palmitoylated derivative of AN1792 was completely non-immunogenic when administered in PBS and gave modest titers when presented in an o/w emulsion with GMTs of 340 and 105 for the third and fourth bleeds. The highest antibody titers were generated with Freund's adjuvant with a peak GMT of about 87,000, a value almost 30-fold greater than the GMTs of the next two most potent vaccines, MPL and high dose AN1792/alum.

The most promising adjuvants identified in this study are MPL and alum. Of these two, MPL appears preferable because a 10-fold lower antigen dose was required to generate the same antibody response as obtained with alum. The response can be increased by increasing the dose of antigen and /or adjuvant and by optimizing the immunization schedule. The o/w emulsion

was a very weak adjuvant for AN1792 and adding an o/w emulsion to MPL adjuvant diminished the intrinsic adjuvant activity of MPL alone.

## 2. A $\beta$ Levels In The Brain

At about 14 weeks the guinea pigs were deeply anesthetized, the cerebrospinal fluid (CSF) was drawn and brains were excised from animals in a subset of the groups, those immunized with Freund's adjuvant (group 2), MPL (group 5), alum with a high dose, 300  $\mu$ g, of AN1792 (group 10) and the PBS immunized control group (group 3). To measure the level of A $\beta$  peptide, one hemisphere was dissected and homogenates of the hippocampal, cortical, and cerebellar regions were prepared in 5 M guanidine. These were diluted and quantitated by comparison to a series of dilutions of A $\beta$  standard protein of known concentrations in an ELISA format. The levels of A $\beta$  protein in the hippocampus, the cortex and the cerebellum were very similar for all four groups despite the wide range of antibody responses to A $\beta$  elicited by these vaccines. Mean A $\beta$  levels of about 25 ng/g tissue were measured in the hippocampus, 21 ng/g in the cortex, and 12 ng/g in the cerebellum. Thus, the presence of a high circulating antibody titer to A $\beta$  for almost three months in some of these animals did not alter the total A $\beta$  levels in their brains. The levels of A $\beta$  in the CSF were also quite similar between the groups. The lack of large effect of AN1792 immunization on endogenous A $\beta$  indicates that the immune response is focused on pathological formations of A $\beta$ .

## VIII. Immune Response to Different Adjuvants in Mice

Six-week old female Swiss Webster mice were used for this study with 10-13 animals per group. Immunizations were given

on days 0, 14, 28, 60, 90 and 20 administered subcutaneously in a dose volume of 200  $\mu$ l. PBS was used as the buffer for all formulations. Animals were bleed seven days following each immunization starting after the second dose for analysis of antibody titers by ELISA. The treatment regime of each group is summarized in Table 7.

Table 7

| Experimental Design of Betabloc Study 010 |                |                        |             |         |           |
|-------------------------------------------|----------------|------------------------|-------------|---------|-----------|
| Group                                     | N <sup>a</sup> | Adjuvant <sup>b</sup>  | Dose        | Antigen | Dose (μg) |
| 1                                         | 10             | MPL                    | 12.5 μg     | AN1792  | 33        |
| 2                                         | 10             | MPL                    | 25 μg       | AN1792  | 33        |
| 3                                         | 10             | MPL                    | 50 μg       | AN1792  | 33        |
| 4                                         | 13             | MPL                    | 125 μg      | AN1792  | 33        |
| 5                                         | 13             | MPL                    | 50 μg       | AN1792  | 150       |
| 6                                         | 13             | MPL                    | 50 μg       | AN1528  | 33        |
| 7                                         | 10             | PBS                    |             | AN1792  | 33        |
| 8                                         | 10             | PBS                    |             | none    |           |
| 9                                         | 10             | Squalene<br>emulsified | 5%          | AN1792  | 33        |
| 10                                        | 10             | Squalene<br>admixed    | 5%          | AN1792  | 33        |
| 11                                        | 10             | Alum                   | 2 mg        | AN1792  | 33        |
| 12                                        | 13             | MPL + Alum             | 50 μg/2 mg  | AN1792  | 33        |
| 13                                        | 10             | QS21                   | 5 μg        | AN1792  | 33        |
| 14                                        | 10             | QS21                   | 10 μg       | AN1792  | 33        |
| 15                                        | 10             | QS21                   | 25 μg       | AN1792  | 33        |
| 16                                        | 13             | QS21                   | 25 μg       | AN1792  | 150       |
| 17                                        | 13             | QS21                   | 25 μg       | AN1528  | 33        |
| 18                                        | 13             | QS21 + MPL             | 25 μg/50 μg | AN1792  | 33        |
| 19                                        | 13             | QS21 + Alum            | 25 μg/2 mg  | AN1792  | 33        |

Footnotes:

<sup>a</sup> Number of mice in each group at the initiation of the experiment.<sup>b</sup> The adjuvants are noted. The buffer for all these formulations was PBS. For group 8, there was no adjuvant and no antigen.

The ELISA titers of antibodies against Aβ42 in each group are shown in Table 8 below.

Table 8.

| Geometric Mean Antibody Titers |       |       |       |       |       |
|--------------------------------|-------|-------|-------|-------|-------|
| Week of Bleed                  |       |       |       |       |       |
| Treatment Group                | 2.9   | 5.0   | 8.7   | 12.9  | 16.7  |
| 1                              | 248   | 1797  | 2577  | 6180  | 4177  |
| 2                              | 598   | 3114  | 3984  | 5287  | 6878  |
| 3                              | 1372  | 5000  | 7159  | 12333 | 12781 |
| 4                              | 1278  | 20791 | 14368 | 20097 | 25631 |
| 5                              | 3288  | 26242 | 13229 | 9315  | 23742 |
| 6                              | 61    | 2536  | 2301  | 1442  | 4504  |
| 7                              | 37    | 395   | 484   | 972   | 2149  |
| 8                              | 25    | 25    | 25    | 25    | 25    |
| 9                              | 29    | 183   | 744   | 952   | 1823  |
| 10                             | 25    | 89    | 311   | 513   | 817   |
| 11                             | 29    | 708   | 2618  | 2165  | 3666  |
| 12                             | 198   | 1458  | 1079  | 612   | 797   |
| 13                             | 38    | 433   | 566   | 1080  | 626   |
| 14                             | 104   | 541   | 3247  | 1609  | 838   |
| 15                             | 212   | 2630  | 2472  | 1224  | 1496  |
| 16                             | 183   | 2616  | 6680  | 2085  | 1631  |
| 17                             | 28    | 201   | 375   | 222   | 1540  |
| 18                             | 31699 | 15544 | 23095 | 6412  | 9059  |
| 19                             | 63    | 243   | 554   | 299   | 441   |

The table shows that the highest titers were obtained for groups 4, 5 and 18, in which the adjuvants were 125  $\mu$ g MPL, 50  $\mu$ g MPL and QS21 plus MPL.

#### IX. Therapeutic Efficacy of Different Adjuvants

A therapeutic efficacy study was conducted in PDAPP transgenic mice with a set of adjuvants suitable for use in humans to determine their ability to potentiate immune responses to A $\beta$  and to induce the immune-mediated clearance of amyloid deposits in the brain.



One hundred eighty male and female, 7.5- to 8.5-month old heterozygous PDAPP transgenic mice were obtained from Charles River Laboratories. The mice were sorted into nine groups containing 15 to 23 animals per group to be immunized with AN1792 or AN1528 combined with various adjuvants. Animals were distributed to match the gender, age, and parentage of the animals within the groups as closely as possible. The adjuvants included alum, MPL, and QS21, each combined with both antigens, and Freund's adjuvant (FA) combined with only AN1792. An additional group was immunized with AN1792 formulated in PBS buffer plus the preservative thimerosal without adjuvant. A ninth group was immunized with PBS alone as a negative control.

Preparation of aggregated A $\beta$  peptides: human A $\beta$ 1-40 (AN1528; California Peptides Inc., Napa, CA; Lot ME0541) and human A $\beta$ 1-42 (AN1792; California Peptides Inc., Lot ME0439) peptides were freshly solubilized for the preparation of each set of injections from lyophilized powders that had been stored desiccated at -20°C. For this purpose, two mg of peptide were added to 0.9 ml of deionized water and the mixture was vortexed to generate a relatively uniform solution or suspension. AN1528 was soluble at this step, in contrast to AN1792. A 100  $\mu$ l aliquot of 10X PBS (1X PBS: 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5) was then added at which point AN1528 began to precipitate. The suspensions were vortexed again and incubated overnight at 37°C for use the next day.

To prepare vaccine doses with alum (Groups 1 and 5), A $\beta$  peptide in PBS was added to Alhydrogel (two percent aqueous aluminum hydroxide gel, Sargeant, Inc., Clifton, NJ) to reach concentrations of 100  $\mu$ g A $\beta$  peptide per 1 mg of alum. 10X PBS was added to a final dose volume of 200  $\mu$ l in 1X PBS. The suspension was then gently mixed for approximately 4 hr at RT prior to injection.

To prepare vaccine doses for with MPL (Groups 2 and 6), lyophilized powder (Ribi ImmunoChem Research, Inc., Hamilton,

MT; Lot 67039-E0896B) was added to 0.2% aqueous triethylamine to a final concentration of 1 mg/ml and vortexed. The mixture was heated to 65 to 70°C for 30 sec to create a slightly opaque uniform suspension of micelles. The solution was stored at 4°C. For each set of injections, 100 µg of peptide per dose in 50 µl PBS, 50 µg of MPL per dose (50 µl) and 100 µl of PBS per dose were mixed in a borosilicate tube immediately before use.

To prepare vaccine doses with QS21 (Groups 3 and 7), lyophilized powder (Aquila, Framingham, MA; Lot A7018R) was added to PBS, pH 6.6-6.7 to a final concentration of 1 mg/ml and vortexed. The solution was stored at -20°C. For each set of injections, 100 µg of peptide per dose in 50 µl PBS, 25 µg of QS21 per dose in 25 µl PBS and 125 µl of PBS per dose were mixed in a borosilicate tube immediately before use.

To prepare vaccine doses with Freund's Adjuvant (Group 4), 100 µg of AN1792 in 200 µl PBS was emulsified 1:1 (vol:vol) with Complete Freund's Adjuvant (CFA) in a final volume of 400 µl for the first immunization. For subsequent immunizations, the antigen was similarly emulsified with Incomplete Freund's Adjuvant (IFA). For the vaccines containing the adjuvants alum, MPL or QS21, 100 µg per dose of AN1792 or AN1528 was combined with alum (1 mg per dose) or MPL (50 µg per dose) or QS21 (25 µg per dose) in a final volume of 200 µl PBS and delivered by subcutaneous inoculation on the back between the shoulder blades. For the group receiving FA, 100 µg of AN1792 was emulsified 1:1 (vol:vol) with Complete Freund's adjuvant (CFA) in a final volume of 400 µl and delivered intraperitoneally for the first immunization, followed by a boost of the same amount of immunogen in Incomplete Freund's adjuvant (IFA) for the subsequent five doses. For the group receiving AN1792 without adjuvant, 10 µg AN1792 was combined with 5 µg thimerosal in a final volume of 50 µl PBS and delivered subcutaneously. The ninth, control group received only 200 µl PBS delivered subcutaneously. Immunizations were given on a biweekly schedule for the first three doses, then

on a monthly schedule thereafter on days 0, 16, 28, 56, 85 and 112. Animals were bled six to seven days following each immunization starting after the second dose for the measurement of antibody titers. Animals were euthanized approximately one week after the final dose. Outcomes were measured by ELISA assay of A $\beta$  and APP levels in brain and by immunohistochemical evaluation of the presence of amyloid plaques in brain sections. In addition, A $\beta$ -specific antibody titers, and A $\beta$ -dependent proliferative and cytokine responses were determined.

Table 9 shows that the highest antibody titers to A $\beta$ 1-42 were elicited with FA and AN1792, titers which peaked following the fourth immunization (peak GMT: 75,386) and then declined by 59% after the final, sixth immunization. The peak mean titer elicited by MPL with AN1792 was 62% lower than that generated with FA (peak GMT: 28,867) and was also reached early in the immunization scheme, after 3 doses, followed by a decline to 28% of the peak value after the sixth immunization. The peak mean titer generated with QS21 combined with AN1792 (GMT: 1,511) was about 5-fold lower than obtained with MPL. In addition, the kinetics of the response were slower, since an additional immunization was required to reach the peak response. Titers generated by alum-bound AN1792 were marginally greater than those obtained with QS21 and the response kinetics were more rapid. For AN1792 delivered in PBS with thimerosal the frequency and size of titers were barely greater than that for PBS alone. The peak titers generated with MPL and AN1528 (peak GMT 3099) were about 9-fold lower than those with AN1792. Alum-bound AN1528 was very poorly immunogenic with low titers generated in only some of the animals. No antibody responses were observed in the control animals immunized with PBS alone.

Table 9

| Geometric Mean Antibody Titers <sup>a</sup> |                             |                   |                   |                   |                   |
|---------------------------------------------|-----------------------------|-------------------|-------------------|-------------------|-------------------|
| Week of Bleed                               |                             |                   |                   |                   |                   |
| Treatment                                   | 3.3                         | 5.0               | 9.0               | 13.0              | 17.0              |
| Alum/<br>AN1792                             | 102<br>(12/21) <sup>b</sup> | 1,081<br>(17/20)  | 2,366<br>(21/21)  | 1,083<br>(19/21)  | 572<br>(18/21)    |
| MPL/<br>AN1792                              | 6241<br>(21/21)             | 28,867<br>(21/21) | 1,1242<br>(21/21) | 5,665<br>(20/20)  | 8,204<br>(20/20)  |
| QS21/<br>AN1792                             | 30<br>(1/20)                | 227<br>(10/19)    | 327<br>(10/19)    | 1,511<br>(17/18)  | 1,188<br>(14/18)  |
| CFA/<br>AN1792                              | 10,076<br>(15/15)           | 61,279<br>(15/15) | 75,386<br>(15/15) | 41,628<br>(15/15) | 30,574<br>(15/15) |
| Alum/<br>AN1528                             | 25<br>(0/21)                | 33<br>(1/21)      | 39<br>(3/20)      | 37<br>(1/20)      | 31<br>(2/20)      |
| MPL/<br>AN1528                              | 184<br>(15/21)              | 2,591<br>(20/21)  | 1,653<br>(21/21)  | 1,156<br>(20/20)  | 3,099<br>(20/20)  |
| QS21/<br>AN1528                             | 29<br>(1/22)                | 221<br>(13/22)    | 51<br>(4/22)      | 820<br>(20/22)    | 2,994<br>(21/22)  |
| PBS plus<br>Thimerosal                      | 25<br>(0/16)                | 33<br>(2/16)      | 39<br>(4/16)      | 37<br>(3/16)      | 47<br>(4/16)      |
| PBS                                         | 25<br>(0/16)                | 25<br>(0/16)      | 25<br>(0/15)      | 25<br>(0/12)      | 25<br>(0/16)      |

## Footnotes:

<sup>a</sup> Geometric mean antibody titers measured against A $\beta$ 1-42<sup>b</sup> Number of responders per group

The results of AN1792 or AN1592 treatment with various adjuvants, or thimerosal on cortical amyloid burden in 12-month old mice determined by ELISA are shown in Fig. 15. In PBS control PDAPP mice, the median level of total A $\beta$  in the

cortex at 12 months was 1,817 ng/g. Notably reduced levels of A $\beta$  were observed in mice treated with AN1792 plus CFA/IFA, AN1792 plus alum, AN1792 plus MPL and QS21 plus AN1792. The reduction reached statistical significance ( $p < 0.05$ ) only for AN1792 plus CFA/IFA. However, as shown in Examples I and III, the effects of immunization in reducing A $\beta$  levels become substantially greater in 15 month and 18 month old mice. Thus, it is expected that at least the AN1792 plus alum, AN1792 plus MPL and AN1792 plus QS21 compositions will achieve statistical significance in treatment of older mice. By contrast, the AN1792 plus the preservative thimerosal showed a median level of A $\beta$  about the same as that in the PBS treated mice. Similar results were obtained when cortical levels of A $\beta$ 42 were compared. The median level of A $\beta$ 42 in PBS controls was 1624 ng/g. Notably reduced median levels of 403, 1149, 620 and 714 were observed in the mice treated with AN1792 plus CFA/IFA, AN1792 plus alum, AN1792 plus MPL and AN1792 plus QS21 respectively, with the reduction achieving statistical significance ( $p = 0.05$ ) for the AN1792 CFA/IFA treatment group. The median level in the AN1792 thimerosal treated mice was 1619 ng/g A $\beta$ 42.

#### X. Toxicity Analysis

Tissues were collected for histopathologic examination at the termination of studies described in Examples 2, 3 and 7. In addition, hematology and clinical chemistry were performed on terminal blood samples from Examples 3 and 7. Most of the major organs were evaluated, including brain, pulmonary, lymphoid, gastrointestinal, liver, kidney, adrenal and gonads. Although sporadic lesions were observed in the study animals, there were no obvious differences, either in tissues affected or lesion severity, between AN1792 treated and untreated animals. There were no unique histopathological lesions noted in AN-1782-immunized animals compared to PBS-treated or untreated animals. There were also no differences in the

clinical chemistry profile between adjuvant groups and the PBS treated animals in Example 7. Although there were significant increases in several of the hematology parameters between animals treated with AN1792 and Freund's adjuvant in Example 7 relative to PBS treated animals, these type of effects are expected from Freund's adjuvant treatment and the accompanying peritonitis and do not indicate any adverse effects from AN1792 treatment. Although not part of the toxicological evaluation, PDAPP mouse brain pathology was extensively examined as part of the efficacy endpoints. No sign of treatment related adverse effect on brain morphology was noted in any of the studies. These results indicate that AN1792 treatment is well tolerated and at least substantially free of side effects.

## XI. Prevention and Treatment of Subjects

A single-dose phase I trial is performed to determine safety. A therapeutic agent is administered in increasing dosages to different patients starting from about 0.01 the level of presumed efficacy, and increasing by a factor of three until a level of about 10 times the effective mouse dosage is reached.

A phase II trial is performed to determine therapeutic efficacy. Patients with early to mid Alzheimer's Disease defined using Alzheimer's disease and Related Disorders Association (ADRDA) criteria for probable AD are selected. Suitable patients score in the 12-26 range on the Mini-Mental State Exam (MMSE). Other selection criteria are that patients are likely to survive the duration of the study and lack complicating issues such as use of concomitant medications that may interfere. Baseline evaluations of patient function are made using classic psychometric measures, such as the MMSE, and the ADAS, which is a comprehensive scale for evaluating patients with Alzheimer's Disease status and

function. These psychometric scales provide a measure of progression of the Alzheimer's condition. Suitable qualitative life scales can also be used to monitor treatment. Disease progression can also be monitored by MRI. Blood profiles of patients can also be monitored including assays of immunogen-specific antibodies and T-cells responses.

Following baseline measures, patients begin receiving treatment. They are randomized and treated with either therapeutic agent or placebo in a blinded fashion. Patients are monitored at least every six months. Efficacy is determined by a significant reduction in progression of a treatment group relative to a placebo group.

A second phase II trial is performed to evaluate conversion of patients from non-Alzheimer's Disease early memory loss, sometimes referred to as age-associated memory impairment (AAMI), to probable Alzheimer's disease as defined as by ADRDA criteria. Patients with high risk for conversion to Alzheimer's Disease are selected from a non-clinical population by screening reference populations for early signs of memory loss or other difficulties associated with pre-Alzheimer's symptomatology, a family history of Alzheimer's Disease, genetic risk factors, age, sex, and other features found to predict high-risk for Alzheimer's Disease. Baseline scores on suitable metrics including the MMSE and the ADAS together with other metrics designed to evaluate a more normal population are collected. These patient populations are divided into suitable groups with placebo comparison against dosing alternatives with the agent. These patient populations are followed at intervals of about six months, and the endpoint for each patient is whether or not he or she converts to probable Alzheimer's Disease as defined by ADRDA criteria at the end of the observation.

## XII. General Materials and Methods

### 1. Measurement of Antibody Titers

Mice were bled by making a small nick in the tail vein and collecting about 200  $\mu$ l of blood into a microfuge tube.

5 Guinea pigs were bled by first shaving the back hock area and then using an 18 gauge needle to nick the metatarsal vein and collecting the blood into microfuge tubes. Blood was allowed to clot for one hr at room temperature (RT), vortexed, then centrifuged at 14,000 x g for 10 min to separate the clot from  
10 the serum. Serum was then transferred to a clean microfuge tube and stored at 4° C until titered.

Antibody titers were measured by ELISA. 96-well microtiter plates (Costar EIA plates) were coated with 100  $\mu$ l of a solution containing either 10  $\mu$ g/ml either A $\beta$ 42 or SAPP or  
15 other antigens as noted in each of the individual reports in Well Coating Buffer (0.1 M sodium phosphate, pH 8.5, 0.1% sodium azide) and held overnight at RT. The wells were aspirated and sera were added to the wells starting at a 1/100 dilution in Specimen Diluent (0.014 M sodium phosphate, pH  
20 7.4, 0.15 M NaCl, 0.6% bovine serum albumin, 0.05% thimerosal). Seven serial dilutions of the samples were made directly in the plates in three-fold steps to reach a final dilution of 1/218,700. The dilutions were incubated in the coated-plate wells for one hr at RT. The plates were then  
25 washed four times with PBS containing 0.05% Tween 20. The second antibody, a goat anti-mouse Ig conjugated to horseradish peroxidase (obtained from Boehringer Mannheim), was added to the wells as 100  $\mu$ l of a 1/3000 dilution in Specimen Diluent and incubated for one hr at RT. Plates were  
30 again washed four times in PBS, Tween 20. To develop the chromogen, 100  $\mu$ l of Slow TMB (3,3',5,5'-tetramethyl benzidine obtained from Pierce Chemicals) was added to each well and incubated for 15 min at RT. The reaction was stopped by the addition of 25  $\mu$ l of 2 M H<sub>2</sub>SO<sub>4</sub>. The color intensity was then  
35 read on a Molecular Devices Vmax at (450 nm - 650 nm).



Titers were defined as the reciprocal of the dilution of serum giving one half the maximum OD. Maximal OD was generally taken from an initial 1/100 dilution, except in cases with very high titers, in which case a higher initial dilution was necessary to establish the maximal OD. If the 50% point fell between two dilutions, a linear extrapolation was made to calculate the final titer. To calculate geometric mean antibody titers, titers less than 100 were arbitrarily assigned a titer value of 25.

## 2. Lymphocyte proliferation assay

Mice were anesthetized with isoflurane. Spleens were removed and rinsed twice with 5 ml PBS containing 10% heat-inactivated fetal bovine serum (PBS-FBS) and then homogenized in a 50  $\mu$  Centricon unit (Dako A/S, Denmark) in 1.5 ml PBS-FBS for 10 sec at 100 rpm in a Medimachine (Dako) followed by filtration through a 100  $\mu$  pore size nylon mesh. Splenocytes were washed once with 15 ml PBS-FBS, then pelleted by centrifugation at 200 x g for 5 min. Red blood cells were lysed by resuspending the pellet in 5 mL buffer containing 0.15 M  $\text{NH}_4\text{Cl}$ , 1 M  $\text{KHCO}_3$ , 0.1 M NaEDTA, pH 7.4 for five min at RT. Leukocytes were then washed as above. Freshly isolated spleen cells ( $10^5$  cells per well) were cultured in triplicate sets in 96-well U-bottomed tissue culture-treated microtiter plates (Corning, Cambridge, MA) in RPMI 1640 medium (JRH Biosciences, Lenexa, KS) supplemented with 2.05 mM L glutamine, 1% Penicillin/Streptomycin, and 10% heat-inactivated FBS, for 96 hr at 37°C. Various A $\beta$  peptides, A $\beta$ 1-16, A $\beta$ 1-40, A $\beta$ 1-42 or A $\beta$ 40-1 reverse sequence protein were also added at doses ranging from 5  $\mu\text{M}$  to 0.18  $\mu\text{M}$  in four steps. Cells in control wells were cultured with Concanavalin A (Con A) (Sigma, cat. # C-5275, at 1  $\mu\text{g}/\text{ml}$ ) without added protein. Cells were pulsed for the final 24 hr with  $^3\text{H}$ -thymidine (1  $\mu\text{Ci}/\text{well}$  obtained from Amersham Corp., Arlington Heights IL). Cells were then harvested onto UniFilter plates

and counted in a Top Count Microplate Scintillation Counter (Packard Instruments, Downers Grove, IL). Results are expressed as counts per minute (cpm) of radioactivity incorporated into insoluble macromolecules.

#### 5      4. Brain Tissue Preparation

After euthanasia, the brains were removed and one hemisphere was prepared for immunohistochemical analysis, while three brain regions (hippocampus, cortex and cerebellum) were dissected from the other hemisphere and used to measure the concentration of various A $\beta$  proteins and APP forms using specific ELISAs (Johnson-Wood et al., supra).

Tissues destined for ELISAs were homogenized in 10 volumes of ice-cold guanidine buffer (5.0 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0). The homogenates were mixed by gentle agitation using an Adams Nutator (Fisher) for three to four hr at RT, then stored at -20°C prior to quantitation of A $\beta$  and APP. Previous experiments had shown that the analytes were stable under this storage condition, and that synthetic A $\beta$  protein (Bachem) could be quantitatively recovered when spiked into homogenates of control brain tissue from mouse littermates (Johnson-Wood et al., supra).

#### 5. Measurement of A $\beta$ Levels

The brain homogenates were diluted 1:10 with ice cold Casein Diluent (0.25% casein, PBS, 0.05% sodium azide, 20  $\mu$ g/ml aprotinin, 5 mM EDTA pH 8.0, 10  $\mu$ g/ml leupeptin) and then centrifuged at 16,000 x g for 20 min at 4° C. The synthetic A $\beta$  protein standards (1-42 amino acids) and the APP standards were prepared to include 0.5 M guanidine and 0.1% bovine serum albumin (BSA) in the final composition. The

"total" A $\beta$  sandwich ELISA utilizes monoclonal antibody (mA $\beta$ ) 266, specific for amino acids 13-28 of A $\beta$  (Seubert, et al.), as the capture antibody, and biotinylated mA $\beta$  3D6, specific for amino acids 1-5 of A $\beta$  (Johnson-Wood, et al), as the reporter antibody. The 3D6 mA $\beta$  does not recognize secreted APP or full-length APP, but detects only A $\beta$  species with an amino-terminal aspartic acid. This assay has a lower limit of sensitivity of ~50  $\mu$ g/ml (11  $\mu$ M) and shows no cross-reactivity to the endogenous murine A $\beta$  protein at concentrations up to 1 ng/ml (Johnson-Wood et al., *supra*).

The A $\beta$ 1-42 specific sandwich ELISA employs mA $\beta$  21F12, specific for amino acids 33-42 of A $\beta$  (Johnson-Wood, et al.), as the capture antibody. Biotinylated mA $\beta$  3D6 is also the reporter antibody in this assay which has a lower limit of sensitivity of about 125  $\mu$ g/ml (28  $\mu$ M, Johnson-Wood et al.). For the A $\beta$  ELISAs, 100  $\mu$ l of either mA $\beta$  266 (at 10  $\mu$ g/ml) or mA $\beta$  21F12 at (5  $\mu$ g/ml) was coated into the wells of 96-well immunoassay plates (Costar) by overnight incubation at RT. The solution was removed by aspiration and the wells were blocked by the addition of 200  $\mu$ l of 0.25% human serum albumin in PBS buffer for at least 1 hr at RT. Blocking solution was removed and the plates were stored desiccated at 4°C until used. The plates were rehydrated with Wash Buffer [Tris-buffered saline (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.5), plus 0.05% Tween 20] prior to use. The samples and standards were added in triplicate aliquots of 100  $\mu$ l per well and then incubated overnight at 4° C. The plates were washed at least three times with Wash Buffer between each step of the assay. The biotinylated mA $\beta$  3D6, diluted to 0.5  $\mu$ g/ml in Casein Assay Buffer (0.25% casein, PBS, 0.05% Tween 20, pH 7.4), was added and incubated in the wells for 1 hr at RT. An avidin-horseradish peroxidase conjugate, (Avidin-HRP obtained from Vector, Burlingame, CA), diluted 1:4000 in Casein Assay Buffer, was added to the wells for 1 hr at RT. The colorimetric substrate, Slow TMB-ELISA (Pierce), was added and allowed to react for 15 minutes at RT, after which the enzymatic reaction was stopped by the addition of 25  $\mu$ l 2 N

H<sub>2</sub>SO<sub>4</sub>. The reaction product was quantified using a Molecular Devices Vmax measuring the difference in absorbance at 450 nm and 650 nm.

## 6. Measurement of APP Levels

Two different APP assays were utilized. The first, designated APP- $\alpha$ /FL, recognizes both APP-alpha ( $\alpha$ ) and full-length (FL) forms of APP. The second assay is specific for APP- $\alpha$ . The APP- $\alpha$ /FL assay recognizes secreted APP including the first 12 amino acids of A $\beta$ . Since the reporter antibody (2H3) is not specific to the  $\alpha$ -clip-site, occurring between amino acids 612-613 of APP695 (Esch et al., *Science* 248, 1122-1124 (1990)); this assay also recognizes full length APP (APP-FL). Preliminary experiments using immobilized APP antibodies to the cytoplasmic tail of APP-FL to deplete brain homogenates of APP-FL suggest that approximately 30-40% of the APP- $\alpha$ /FL APP is FL (data not shown). The capture antibody for both the APP- $\alpha$ /FL and APP- $\alpha$  assays is mA $\beta$  8E5, raised against amino acids 444 to 592 of the APP695 form (Games et al., *supra*). The reporter mA $\beta$  for the APP- $\alpha$ /FL assay is mA $\beta$  2H3, specific for amino acids 597-608 of APP695 (Johnson-Wood et al., *supra*) and the reporter antibody for the APP- $\alpha$  assay is a biotinylated derivative of mA $\beta$  16H9, raised to amino acids 605 to 611 of APP. The lower limit of sensitivity of the APP- $\alpha$ /FL assay is about 11 ng/ml (150 pM) (Johnson-Wood et al.) and that of the APP- $\alpha$  specific assay is 22 ng/ml (0.3 nM). For both APP assays, mA $\beta$  8E5 was coated onto the wells of 96-well EIA plates as described above for mA $\beta$  266. Purified, recombinant secreted APP- $\alpha$  was used as the reference standard for the APP- $\alpha$  assay and the APP- $\alpha$ /FL assay (Esch et al., *supra*). The brain homogenate samples in 5 M guanidine were diluted 1:10 in ELISA Specimen Diluent (0.014 M phosphate buffer, pH 7.4, 0.6% bovine serum albumin, 0.05% thimerosal, 0.5 M NaCl, 0.1% NP40). They were then diluted 1:4 in Specimen Diluent containing 0.5 M guanidine. Diluted

homogenates were then centrifuged at 16,000 x g for 15 seconds at RT. The APP standards and samples were added to the plate in duplicate aliquots and incubated for 1.5 hr at RT. The biotinylated reporter antibody 2H3 or 16H9 was incubated with  
5 samples for 1 hr at RT. Streptavidin-alkaline phosphatase (Boehringer Mannheim), diluted 1:1000 in specimen diluent, was incubated in the wells for 1 hr at RT. The fluorescent substrate 4-methyl-umbelliphenyl-phosphate was added for a 30-min RT incubation and the plates were read on a Cytofluor<sup>tm</sup>  
10 2350 fluorimeter (Millipore) at 365 nm excitation and 450 nm emission.

## 7. Immunohistochemistry

Brains were fixed for three days at 4°C in 4% paraformaldehyde in PBS and then stored from one to seven days  
15 at 4°C in 1% paraformaldehyde, PBS until sectioned. Forty-micron-thick coronal sections were cut on a vibratome at RT and stored in cryoprotectant (30% glycerol, 30% ethylene glycol in phosphate buffer) at -20°C prior to immunohistochemical processing. For each brain, six sections  
20 at the level of the dorsal hippocampus, each separated by consecutive 240 µm intervals, were incubated overnight with one of the following antibodies: (1) a biotinylated anti-Aβ (mAβ, 3D6, specific for human Aβ) diluted to a concentration of 2 µg/ml in PBS and 1% horse serum; or (2) a biotinylated  
25 mAβ specific for human APP, 8E5, diluted to a concentration of 3 µg/ml in PBS and 1.0% horse serum; or (3) a mAβ specific for glial fibrillary acidic protein (GFAP; Sigma Chemical Co.) diluted 1:500 with 0.25% Triton X-100 and 1% horse serum, in Tris-buffered saline, pH 7.4 (TBS); or (4) a mAβ specific for  
30 CD11b, MAC-1 antigen, (Chemicon International) diluted 1:100 with 0.25% Triton X-100 and 1% rabbit serum in TBS; or (5) a mAβ specific for MHC II antigen, (Pharmlingen) diluted 1:100 with 0.25% Triton X-100 and 1% rabbit serum in TBS; or (6) a rat mAβ specific for CD 43 (Pharmlingen) diluted 1:100 with 1%

rabbit serum in PBS or (7) a rat mAb specific for CD 45RA (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS; or (8) a rat monoclonal A $\beta$  specific for CD 45RB (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS; or (9) a rat monoclonal A $\beta$  specific for CD 45 (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS; or (10) a biotinylated polyclonal hamster A $\beta$  specific for CD3e (Pharmingen) diluted 1:100 with 1% rabbit serum in PBS or (11) a rat mAb specific for CD3 (Serotec) diluted 1:200 with 1% rabbit serum in PBS; or with (12) a solution of PBS lacking a primary antibody containing 1% normal horse serum.

Sections reacted with antibody solutions listed in 1,2 and 6-12 above were pretreated with 1.0% Triton X-100, 0.4% hydrogen peroxide in PBS for 20 min at RT to block endogenous peroxidase. They were next incubated overnight at 4°C with primary antibody. Sections reacted with 3D6 or 8E5 or CD3e mAbs were then reacted for one hr at RT with a horseradish peroxidase-avidin-biotin-complex with kit components "A" and "B" diluted 1:75 in PBS (Vector Elite Standard Kit, Vector Labs, Burlingame, CA.). Sections reacted with antibodies specific for CD 45RA, CD 45RB, CD 45, CD3 and the PBS solution devoid of primary antibody were incubated for 1 hour at RT with biotinylated anti-rat IgG (Vector) diluted 1:75 in PBS or biotinylated anti-mouse IgG (Vector) diluted 1:75 in PBS, respectively. Sections were then reacted for one hr at RT with a horseradish peroxidase-avidin-biotin-complex with kit components "A" and "B" diluted 1:75 in PBS (Vector Elite Standard Kit, Vector Labs, Burlingame, CA.).

Sections were developed in 0.01% hydrogen peroxide, 0.05% 3,3'-diaminobenzidine (DAB) at RT. Sections destined for incubation with the GFAP-, MAC-1- AND MHC II-specific antibodies were pretreated with 0.6% hydrogen peroxide at RT to block endogenous peroxidase then incubated overnight with the primary antibody at 4°C. Sections reacted with the GFAP antibody were incubated for 1 hr at RT with biotinylated anti-mouse IgG made in horse (Vector Laboratories; Vectastain Elite

ABC Kit) diluted 1:200 with TBS. The sections were next reacted for one hr with an avidin-biotin-peroxidase complex (Vector Laboratories; Vectastain Elite ABC Kit) diluted 1:1000 with TBS. Sections incubated with the MAC-1-or MHC II-specific mAb as the primary antibody were subsequently reacted for 1 hr at RT with biotinylated anti-rat IgG made in rabbit diluted 1:200 with TBS, followed by incubation for one hr with avidin-biotin-peroxidase complex diluted 1:1000 with TBS. Sections incubated with GFAP-, MAC-1- and MHC II-specific antibodies were then visualized by treatment at RT with 0.05% DAB, 0.01% hydrogen peroxide, 0.04% nickel chloride, TBS for 4 and 11 min, respectively.

Immunolabeled sections were mounted on glass slides (VWR, Superfrost slides), air dried overnight, dipped in Propar (Anatech) and overlaid with coverslips using Permount (Fisher) as the mounting medium.

To counterstain A $\beta$  plaques, a subset of the GFAP-positive sections were mounted on Superfrost slides and incubated in aqueous 1% Thioflavin S (Sigma) for 7 min following immunohistochemical processing. Sections were then dehydrated and cleared in Propar, then overlaid with coverslips mounted with Permount.

## 8. Image Analysis

A Videometric 150 Image Analysis System (Oncor, Inc., Gaithersburg, MD) linked to a Nikon Microphot-FX microscope through a CCD video camera and a Sony Trinitron monitor was used for quantification of the immunoreactive slides. The image of the section was stored in a video buffer and a color- and saturation-based threshold was determined to select and calculate the total pixel area occupied by the immunolabeled structures. For each section, the hippocampus was manually outlined and the total pixel area occupied by the hippocampus

was calculated. The percent amyloid burden was measured as: (the fraction of the hippocampal area containing A $\beta$  deposits immunoreactive with mA $\beta$  3D6) x 100. Similarly, the percent neuritic burden was measured as: (the fraction of the hippocampal area containing dystrophic neurites reactive with mA $\beta$  8E5) x100. The C-Imaging System (Compix, Inc., Cranberry Township, PA) operating the Simple 32 Software Application program was linked to a Nikon Microphot-FX microscope through an Optronics camera and used to quantitate the percentage of the retrosplenial cortex occupied by GFAP-positive astrocytes and MAC-1-and MHC II-positive microglia. The image of the immunoreacted section was stored in a video buffer and a monochrome-based threshold was determined to select and calculate the total pixel area occupied by immunolabeled cells. For each section, the retrosplenial cortex (RSC) was manually outlined and the total pixel area occupied by the RSC was calculated. The percent astrocytosis was defined as: (the fraction of RSC occupied by GFAP-reactive astrocytes) X 100. Similarly, percent microgliosis was defined as: (the fraction of the RSC occupied by MAC-1- or MHC II-reactive microglia) X 100. For all image analyses, six sections at the level of the dorsal hippocampus, each separated by consecutive 240  $\mu$ m intervals, were quantitated for each animal. In all cases, the treatment status of the animals was unknown to the observer.

Although the foregoing invention has been described in detail for purposes of clarity of understanding, it will be obvious that certain modifications may be practiced within the scope of the appended claims. All publications and patent documents cited herein are hereby incorporated by reference in their entirety for all purposes to the same extent as if each were so individually denoted.



[illegible]

WHAT IS CLAIMED IS:

- 1        1. A pharmaceutical composition comprising an agent  
2        effective to induce an immunogenic response against A $\beta$  in a  
3        patient, and a pharmaceutically acceptable adjuvant.
- 1        2. The pharmaceutical composition of claim 1, wherein the  
2        agent is A $\beta$  or an active fragment thereof.
- 1        3. The pharmaceutical composition of claim 1 or 2, wherein  
2        the adjuvant comprises alum.
- 1        4. The pharmaceutical composition of claim 1 or 2, wherein  
2        the adjuvant comprises monophosphoryl lipid (MPL).
- 1        5. The pharmaceutical composition of claim 1 or 2, wherein  
2        the adjuvant comprises QS21.
- 1        6. The pharmaceutical composition of any of the preceding  
2        claims, wherein the A $\beta$  or fragment is a component of a  
3        particle.
- 1        7. The pharmaceutical composition of claim 6, wherein the  
2        particle is a polylactide polyglycolide copolymer (PLPG) particle.

1        8. A method of preventing or treating a disease  
2 characterized by amyloid deposit in a patient, comprising:

3        administering an agent effective to induce an immune  
4 response against a peptide component of an amyloid deposit in  
5 the patient.

1        9. The method of claim 8, wherein the amyloid deposit  
2 comprises aggregated A $\beta$  peptide.

1        10. The method of claim 8 or 9, wherein the patient is a  
2 human.

1        11. The method of any of the preceding claims, wherein the  
2 disease is Alzheimer's disease.

1        12. The method of any of the preceding claims, wherein the  
2 patient is asymptomatic.

1        13. The method of any of the preceding claims, wherein  
2 the patient is under 50.

1        14. The method of any of the preceding claims, wherein the  
2 patient has inherited risk factors indicating susceptibility  
3 to Alzheimer's disease.

1        15. The method of any of claims 8-13, wherein the patient  
2        has no known risk factors for Alzheimer's disease.

1        16. The method of any of the preceding claims, wherein the  
2        agent comprises A $\beta$  peptide or an active fragment thereof.

1        17. The method of any of the preceding claims, wherein the  
2        agent is A $\beta$  peptide or an active fragment thereof.

1        18. The method of claim 17, wherein the dose of A $\beta$  peptide  
2        administered to the patient at least 50  $\mu$ g.

1        19. The method of claim 17, wherein the dose of A $\beta$  peptide  
2        administered to the patient is at least 100  $\mu$ g.

1        20. The method of any of the preceding claims, wherein the  
2        A $\beta$  peptide is A $\beta$ 42.

1        21. The method of claim 20, wherein the A $\beta$  peptide is  
2        administered in aggregated form.

1       22. The method of claim any of the preceding claims,  
2 wherein the immune response comprises antibodies that bind to  
3 the A $\beta$  peptide.

1       23. The method of any of the preceding claims, wherein the  
2 immune response comprises T-cells that bind to the A $\beta$  peptide  
3 as a component of an MHC I or MHC II complex.

1       24. The method of any one of claims 8, or 10-15 wherein  
2 agent is an antibody to A $\beta$  which induces an immune response by  
3 binding to A $\beta$  in the patient.

1       25. The method of claims 8 or 10-15, wherein T-cells are  
2 removed from the patient, contacted with A $\beta$  peptide under  
3 conditions in which the T-cells are primed, and the primed T-  
4 cells are administered to the patient.

1       26. The method of any of the preceding claims, wherein the  
2 agent is administered orally, subcutaneously, intramuscularly,  
3 topically or intravenously.

1       27. The method of any of the preceding claims, wherein the  
2 agent is administered intramuscularly or subcutaneously.

1        28. The method of any of the preceding claims, further  
2 comprising screening a library of compounds to identify a  
3 compound reactive with antibodies to A $\beta$ , and administering the  
4 compound to the patient to induce the immune response.

1        29. The method of any one of claims 8, 10-15, 26 or 27,  
2 wherein the agent is an effective dose of a nucleic acid  
3 encoding A $\beta$  or an active fragment thereof, whereby the nucleic  
4 acid is expressed in the patient to produce A $\beta$  or the active  
5 fragment thereof, which induces the immune response.

1        30. The method of claim 29, wherein the nucleic acid is  
2 administered through the skin.

1        31. The method of claim 30, wherein the nucleic acid is  
2 applied to the skin by a patch.

1        32. The method of any of the preceding claims, further  
2 comprising monitoring the patient for the immune response.

1        33. The method of any of the preceding claims, further  
2 comprising administering an adjuvant that enhances the immune  
3 response to the A $\beta$  peptide.

1        34. The method of claim 33, wherein the adjuvant and the  
2 agent are administered together as a composition.

1        35. The method of claim 33, wherein the adjuvant is  
2 administered before the agent.

1        36. The method of claim 33, wherein the adjuvant is  
2 administered after the agent.

1        37. The method of any one of claims 33-36, wherein the  
2 adjuvant is alum.

1        38. The method of any one of claims 33-36, wherein the  
2 adjuvant is MPL.

1        39. The method of any one of claims 33-36, wherein the  
2 adjuvant is QS21.

1        40. The method of anyone of claims 33-36, wherein the dose  
2 of A $\beta$  peptide is greater than 10  $\mu$ g.

1        41. A method of preventing or treating Alzheimer's disease  
2 comprising administering an effective dose of A $\beta$  peptide to a  
3 patient.

1       42. Use of A $\beta$  peptide, or an antibody thereto, in the  
2 manufacture of a medicament for prevention or treatment of  
3 Alzheimer's disease.

1       43. The use of claim 42, wherein the A $\beta$  peptide is  
2 combined with a pharmaceutically acceptable adjuvant in the  
3 manufacture of the medicament.

1       44. A composition comprising A $\beta$  or a fragment linked to a  
2 conjugate molecule that promotes delivery of A $\beta$  to the  
3 bloodstream of a patient and/or promotes an immune response  
4 against A $\beta$ .

1       45. The composition of claim 44, wherein the conjugates  
2 promotes an immune response against A $\beta$ .

1       46. The composition of claim 44 or 45, wherein the  
2 conjugate is cholera toxin.

1       47. The composition of claim 44 or 45, wherein the  
2 conjugate is an immunoglobulin.

1       48. The composition of claim 44 or 45, wherein the  
2 conjugate is attenuated diphtheria toxin CRM 197.



1        49. A pharmaceutical composition comprising an agent  
2 effect to induce an immunogenic response against A $\beta$  in a  
3 patient with the proviso that the composition is free of  
4 Complete Freund's adjuvant.

1        50. A composition comprising a viral vector encoding A $\beta$  or  
2 a fragment thereof effective to induce an immune response  
3 against A $\beta$ .

1        51. A composition of claim 50, wherein the viral vector is  
2 herpes, adenovirus, adenoassociated virus, a retrovirus,  
3 sindbis, semiliki forest virus, vaccinia or avian pox.

1        52. A method of assessing efficacy of an Alzheimer's  
2 treatment method in a patient, comprising

3        determining a baseline amount of antibody specific for A $\beta$   
4 peptide in tissue sample from the patient before treatment  
5 with an agent,

6        comparing an amount of antibody specific for A $\beta$  peptide in  
7 the tissue sample from the patient after treatment with the  
8 agent to the baseline amount of A $\beta$  peptide-specific antibody,

9        wherein an amount of A $\beta$  peptide-specific antibody measured  
10 after the treatment that is significantly greater than the  
11 baseline amount of A $\beta$  peptide-specific antibody indicates a  
12 positive treatment outcome.

1        53. The method of claim 52, wherein the amounts of  
2        antibody are measured as antibody titers.

1        54. The method of claim 53, wherein the amounts of  
2        antibody are measured by an ELISA assay.

1        55. A method of assessing efficacy of an Alzheimer's  
2        treatment method in a patient, comprising

3        determining a baseline amount of antibody specific for A $\beta$   
4        peptide in tissue sample from a patient before treatment with  
5        an agent;

6        comparing an amount of antibody specific for A $\beta$  peptide in  
7        the tissue sample from the subject after treatment with the  
8        agent to the baseline amount of A $\beta$  peptide-specific antibody,

9        wherein a reduction or lack of significant difference  
10       between the amount of A $\beta$  peptide-specific antibody measured  
11       after the treatment compared to the baseline amount of A $\beta$   
12       peptide-specific antibody indicates a negative treatment  
13       outcome.

1        56. A method of assessing efficacy of an Alzheimer's  
2        treatment method in a patient, comprising

3        determining a control amount of antibody specific for A $\beta$   
4        peptide in tissue samples from a control population,

5        comparing an amount of antibody specific for A $\beta$  peptide in  
6        a tissue sample from the patient after administering an agent  
7        to the control amount of A $\beta$  peptide-specific antibody,

8 wherein an amount of A $\beta$  peptide-specific antibody measured  
9 after the treatment that is significantly greater than the  
10 control amount of A $\beta$  peptide-specific antibody indicates a  
11 positive treatment outcome.

1 57. A method of assessing efficacy of an Alzheimer's  
2 treatment method in a patient, comprising

3 determining a control amount of antibody specific for A $\beta$   
4 peptide in tissues samples from a control population,

5 comparing an amount of antibody specific for A $\beta$  peptide in  
6 a tissue sample from the patient after administering an agent  
7 to said control amount of A $\beta$  peptide-specific antibody,

8 wherein a lack of significant difference between the amount  
9 of A $\beta$  peptide-specific antibody measured after beginning said  
10 treatment compared to the control amount of A $\beta$   
11 peptide-specific antibody indicates a negative treatment  
12 outcome.

1 .58. A method of monitoring Alzheimer's disease or  
2 susceptibility thereto in a patient, comprising:

3 detecting an immune response against A $\beta$  peptide in a sample  
4 from the patient.

1 59. The method of claim 58, wherein the patient is being  
2 administered an agent effective to treat or prevent  
3 Alzheimer's disease, and the level of the response determines  
4 the future treatment regime of the patient.

1       60. The method of claim 59, wherein the agent is A $\beta$   
2 peptide.

1       61. The method of any one of claims 57-60, wherein the  
2 detecting comprises detecting an antibody that specifically  
3 binds to A $\beta$  peptide.

1       62. The method of any one of claims 57-60, wherein the  
2 detecting comprises detecting T-cells specifically reactive  
3 with A $\beta$  peptide.

1       63. A method of assessing efficacy of an Alzheimer's  
2 treatment method in a patient, comprising

3       determining a value for an amount of antibody specific for  
4 A $\beta$  peptide in tissue sample from a patient who has been  
5 treated with an agent;

6       comparing the value with a control value determined from a  
7 population of patient experiencing amelioration of, or  
8 freedom from, symptoms of Alzheimer's disease due to treatment  
9 with the agent;

10       wherein a value in the patient at least equal to the  
11 control value indicates a positive response to treatment.

1        64. Use of A $\beta$  peptide in monitoring treatment of  
2 Alzheimer's disease in a patient.

1        65. A diagnostic kit for monitoring treatment of  
2 Alzheimer's disease, comprising:

3        an agent that binds to antibodies specific for A $\beta$  peptide.

1        66. The diagnostic kit of claim 65, further comprising  
2 labelling indicating how the kit is used for monitoring  
3 treatment of Alzheimer's disease.

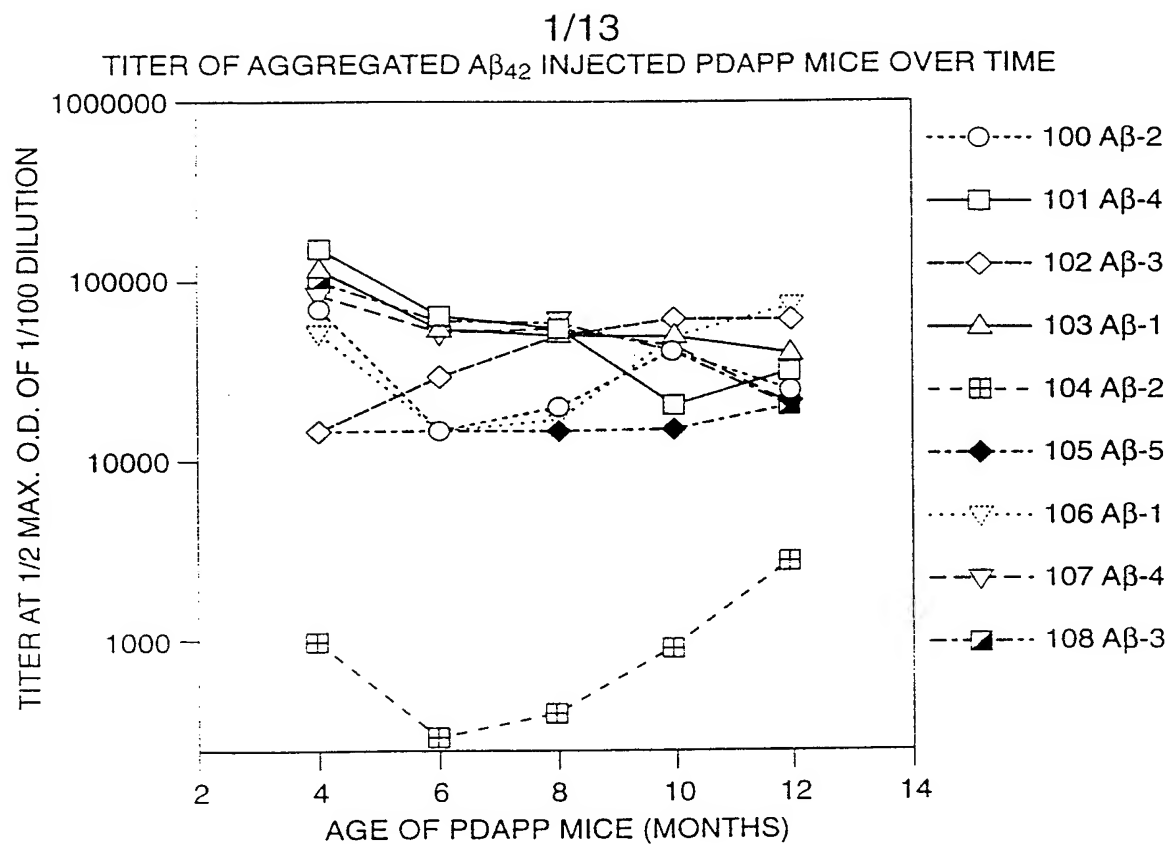


FIG. 1

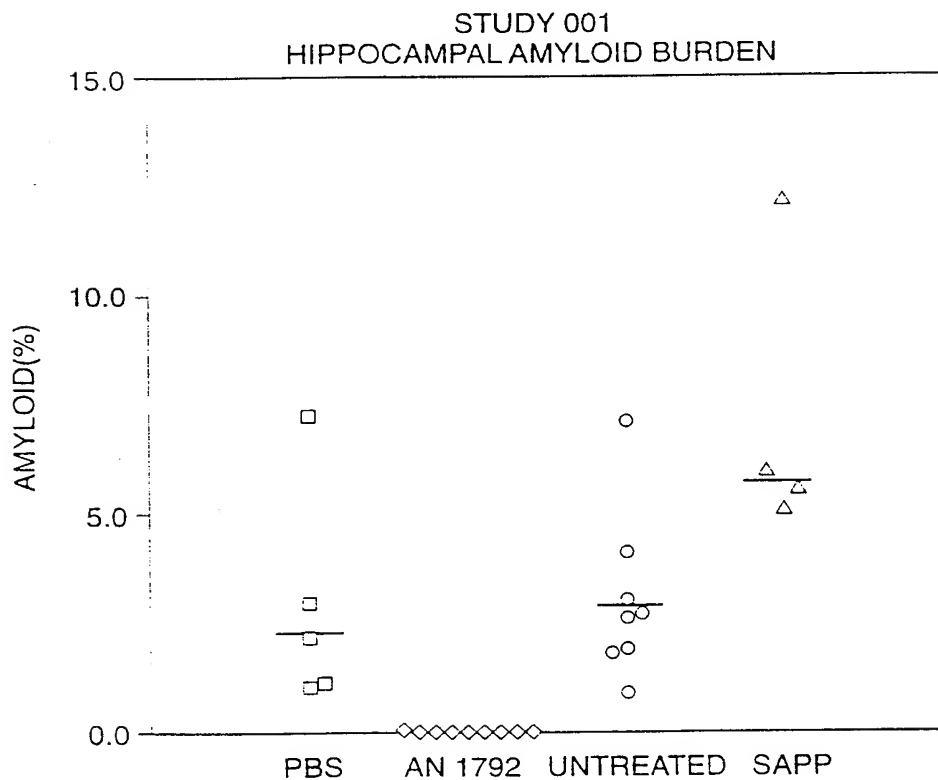


FIG. 2

2/13

STUDY 001

HIPPOCAMPAL NEURITIC PLAQUE BURDEN

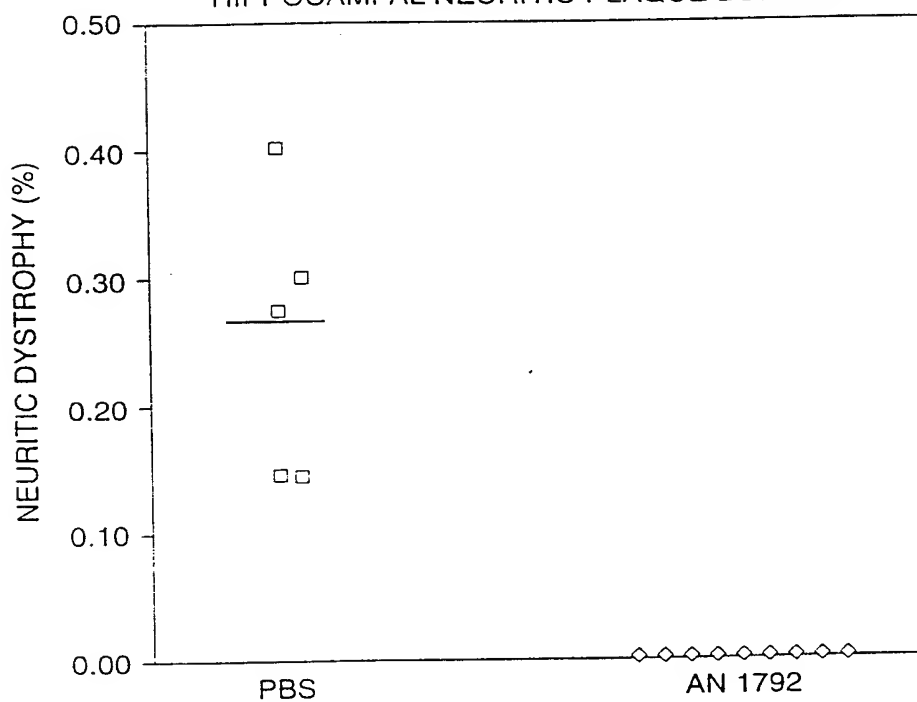


FIG. 3

STUDY 001

# RETROPELENIAL CORTICAL ASTROCYTOSIS

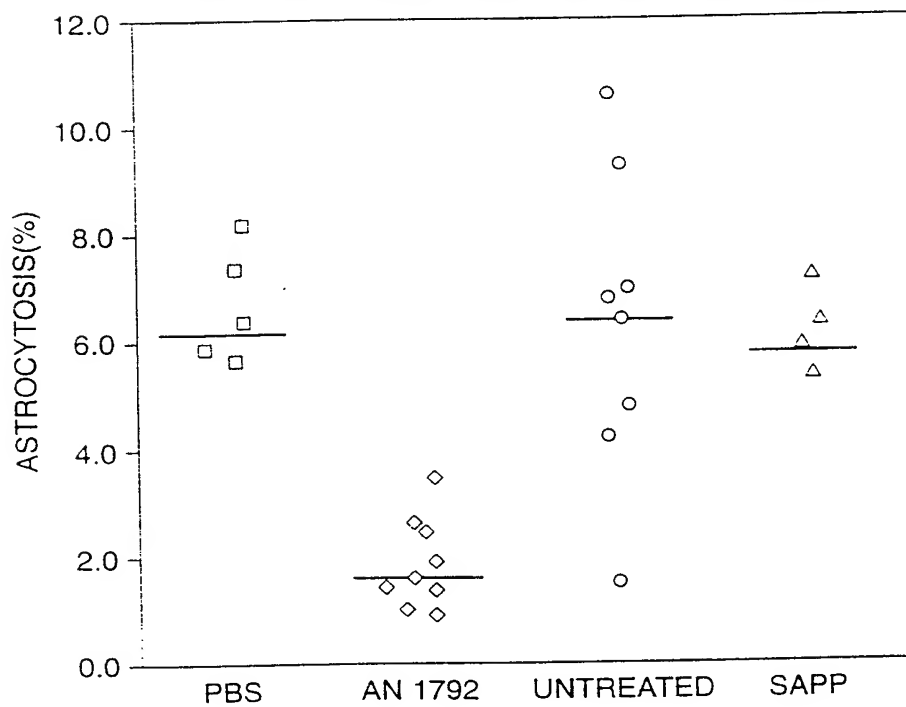


FIG. 4

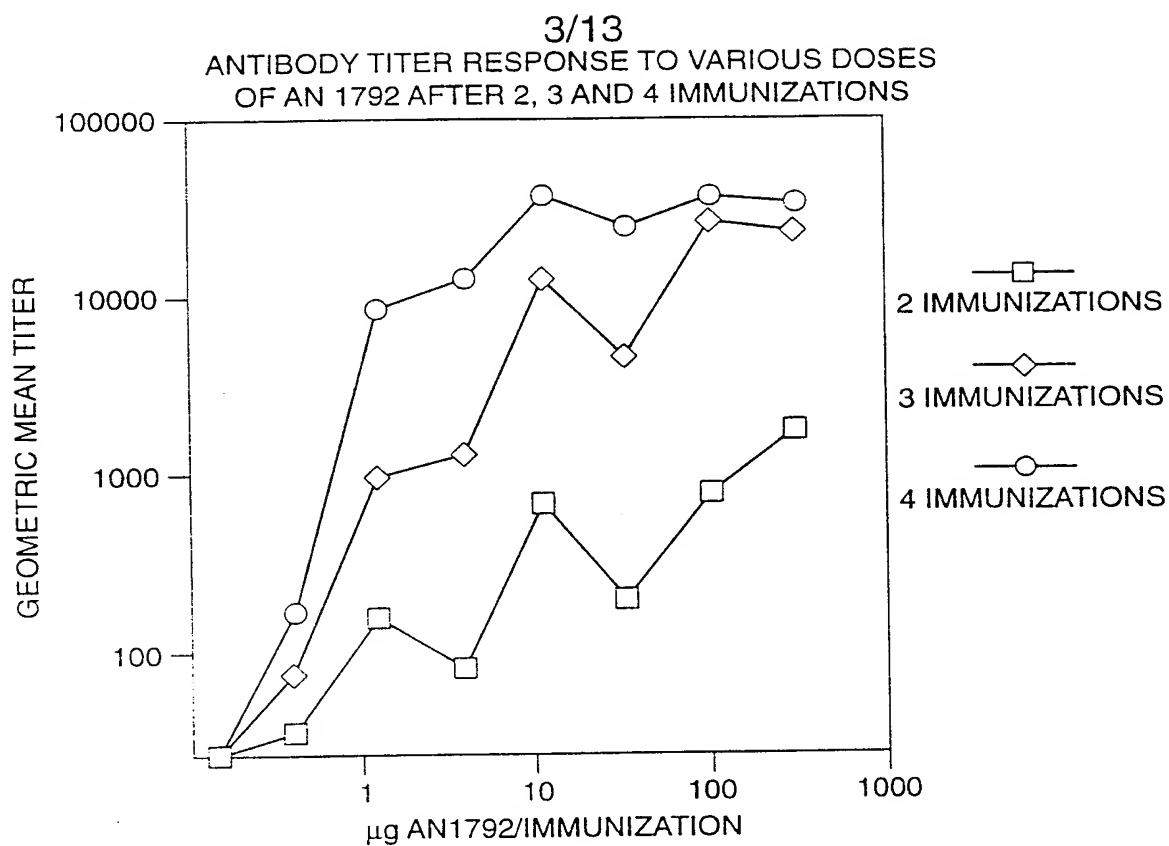


FIG. 5

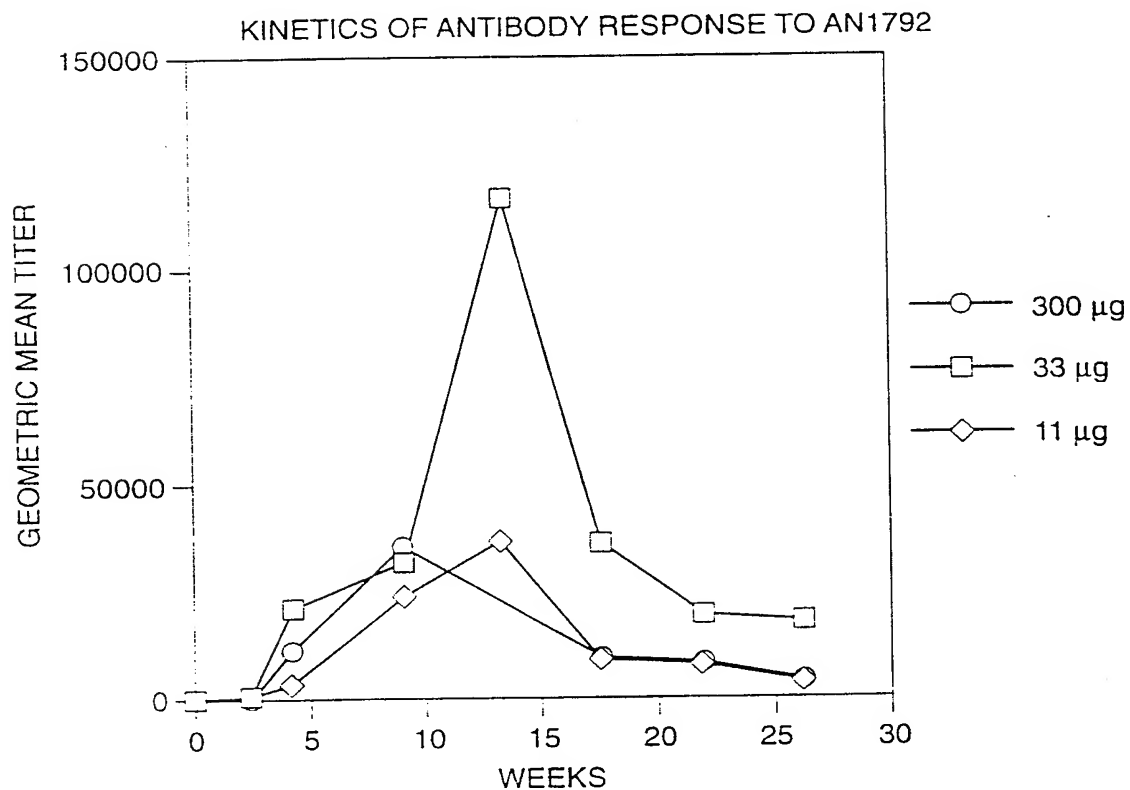


FIG. 6



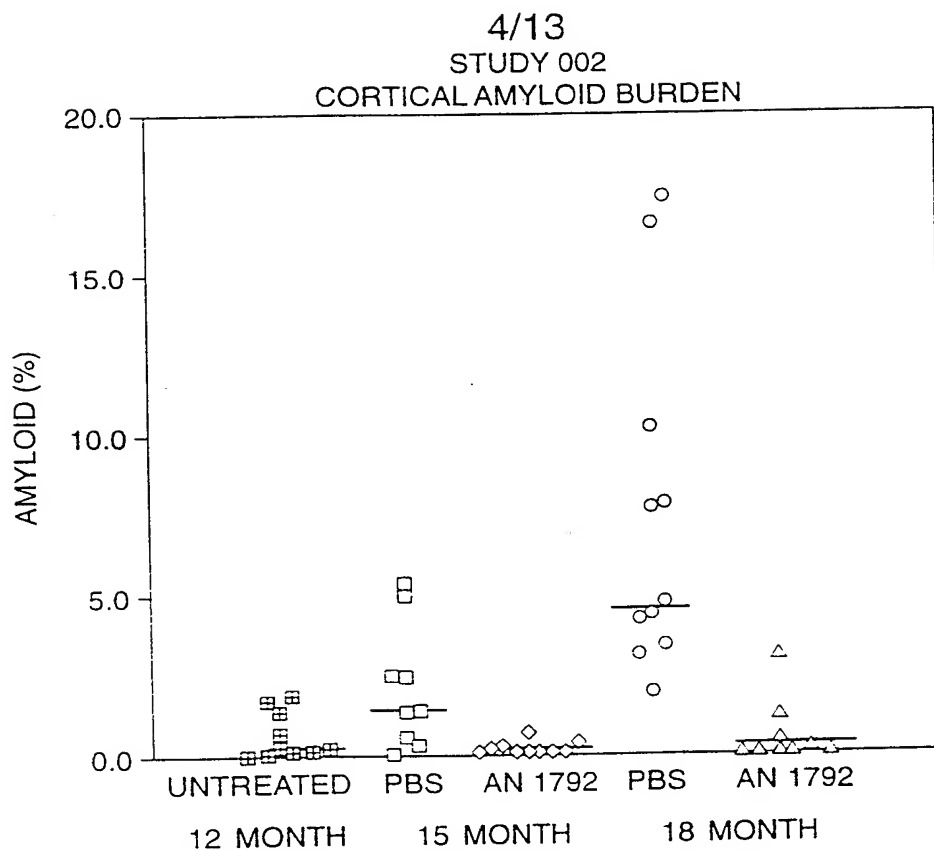


FIG. 7

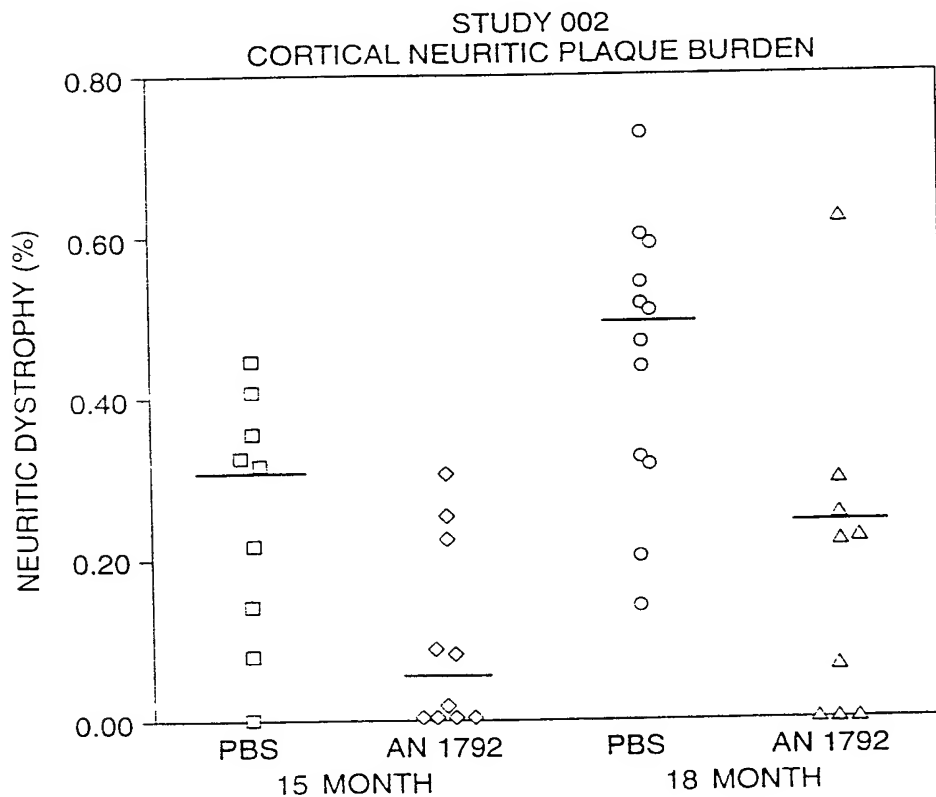


FIG. 8

5/13

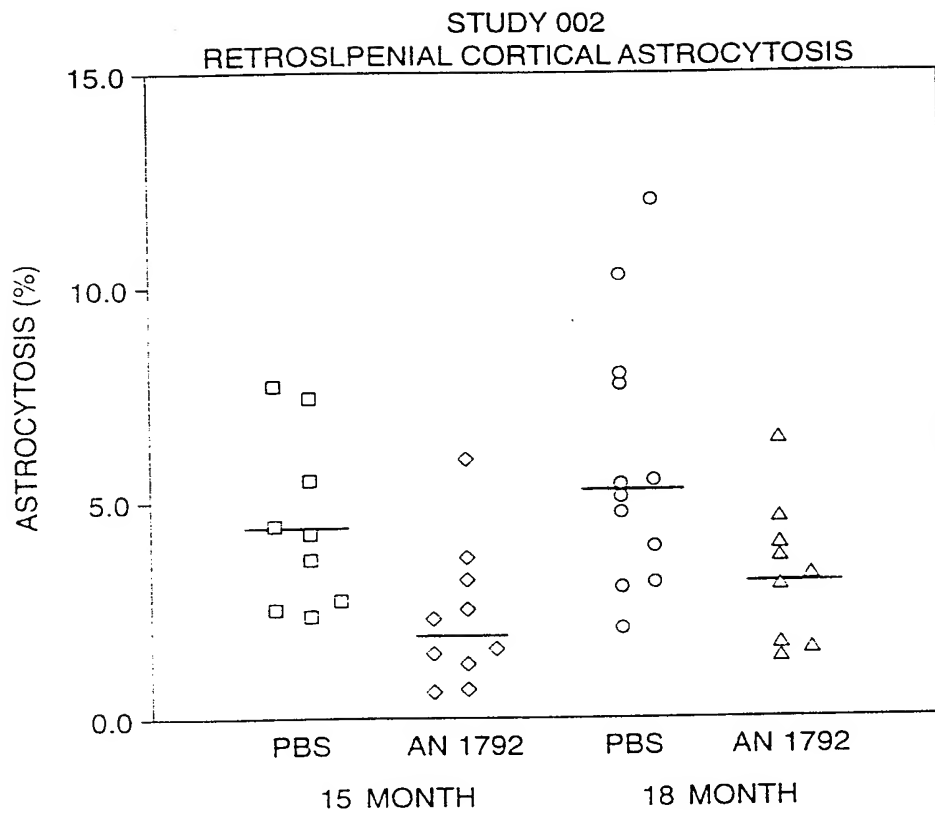


FIG. 9

6/13

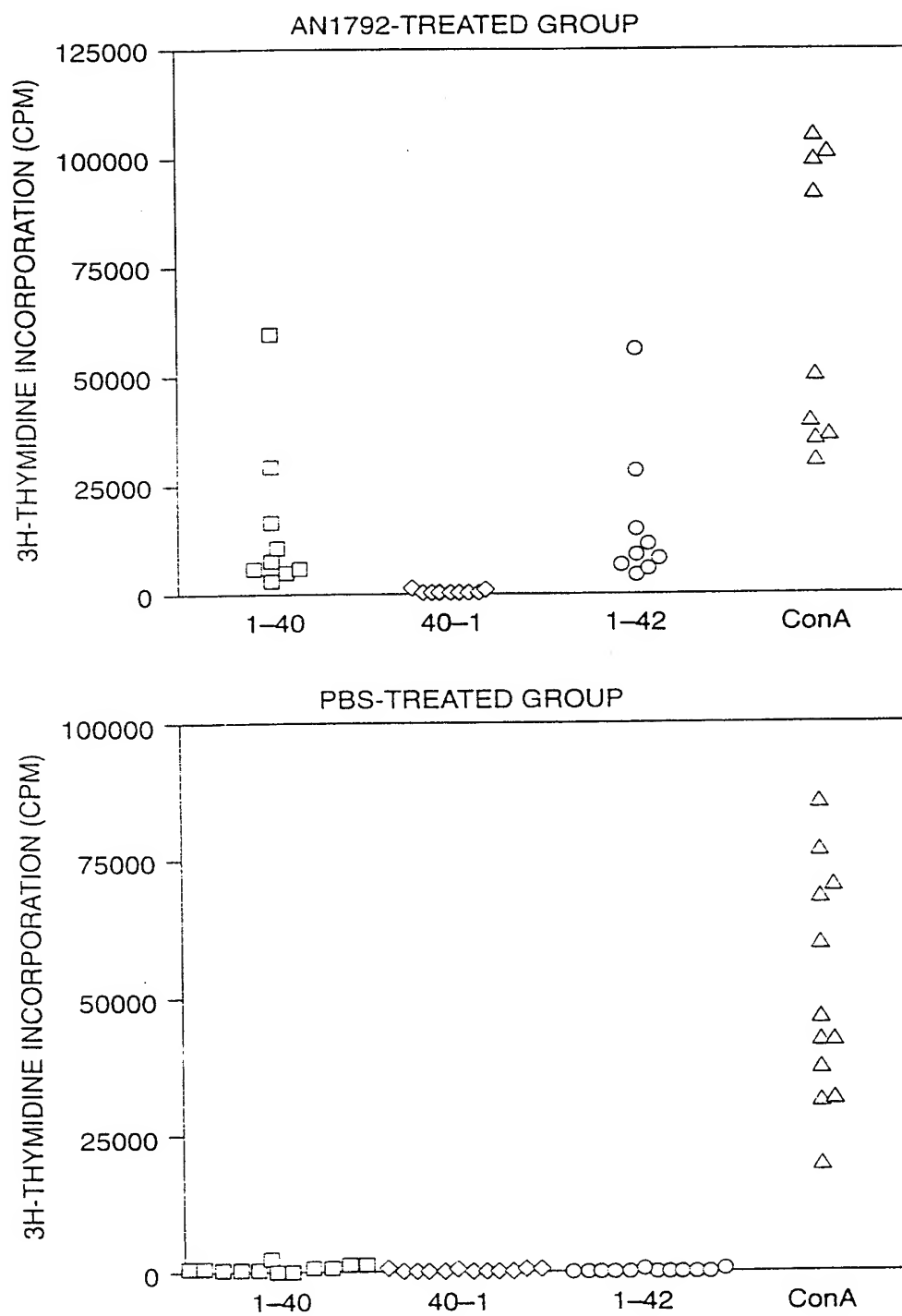
STUDY 002  
RETROSLPENIAL CORTICAL ASTROCYTOSIS

FIG. 10

SUBSTITUTE SHEET (RULE 26)

7/13

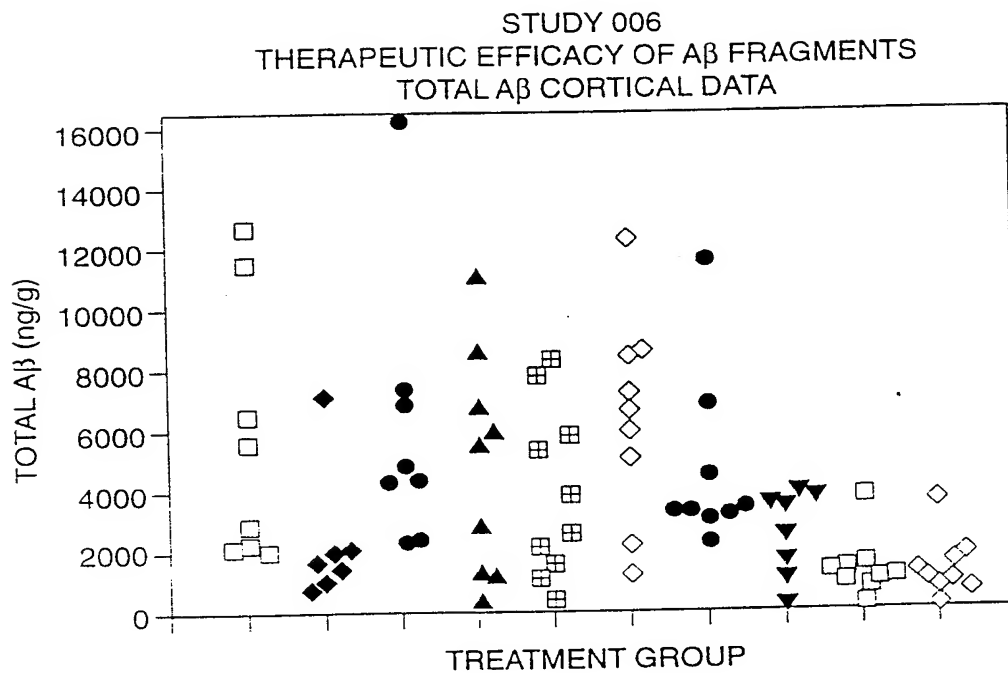


FIG. 11

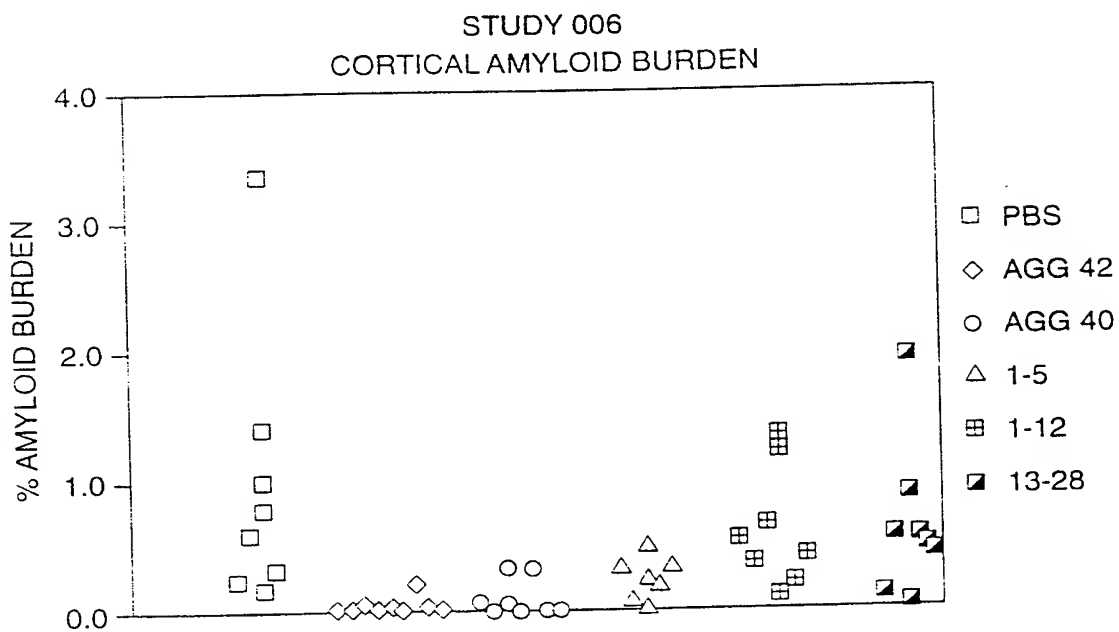


FIG. 12

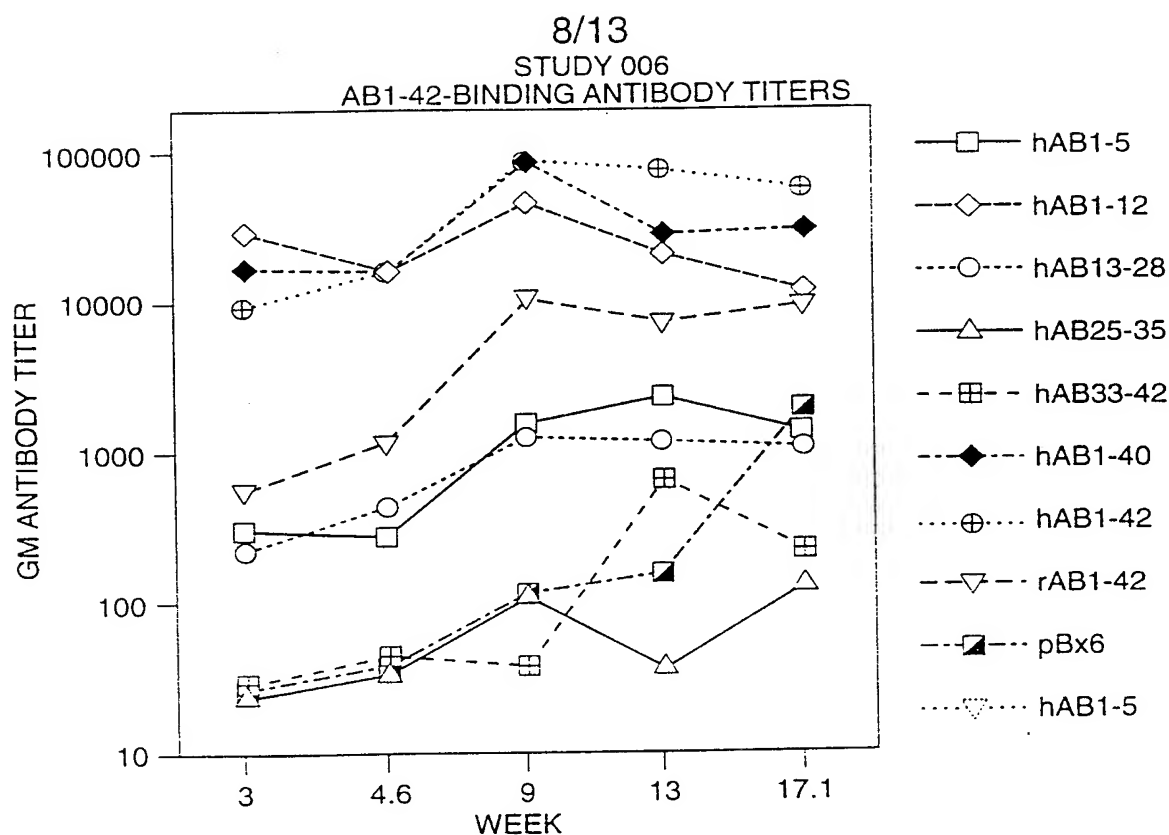


FIG. 13

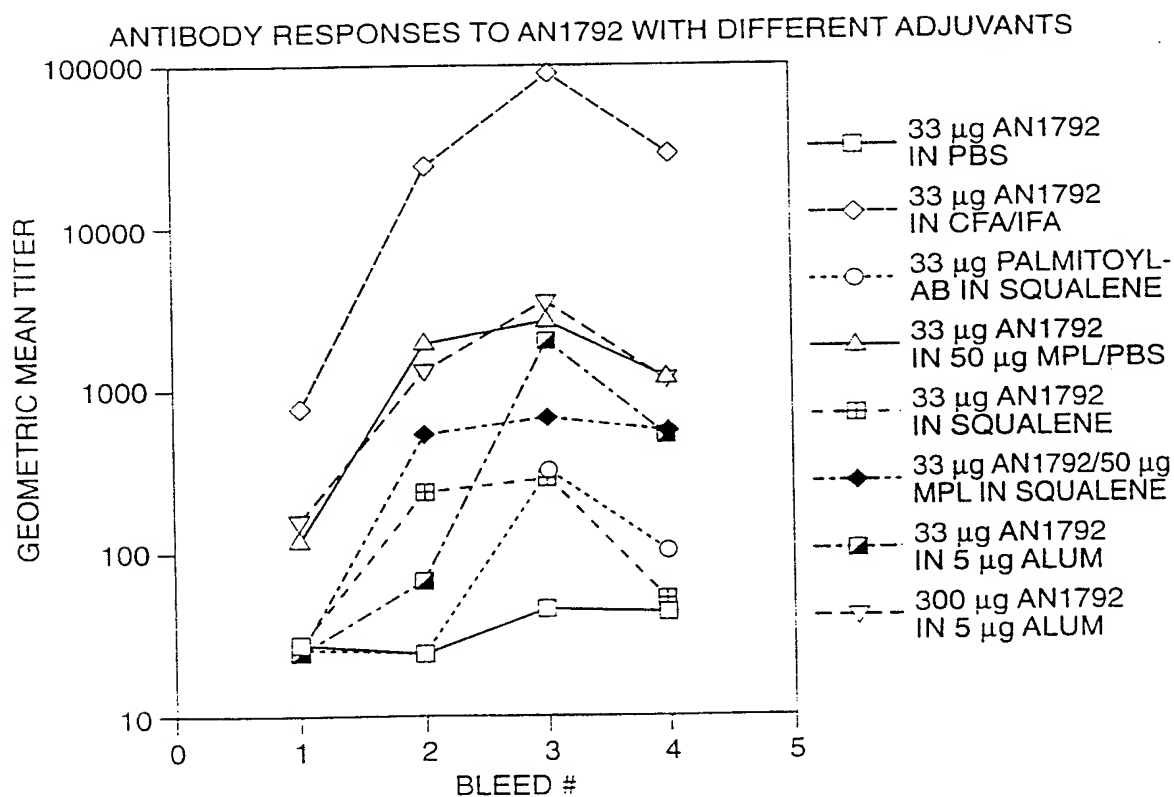


FIG. 14

9/13

## CORTEX

| PBS CONTROL                                  |                            | UNTREATED CONTROL                            |                            |
|----------------------------------------------|----------------------------|----------------------------------------------|----------------------------|
| 624-165                                      | 272                        | 764-181                                      | 3470                       |
| 625-166                                      | 1802                       | 785-182                                      | 171                        |
| 626-167                                      | 62                         | 766-183                                      | 91                         |
| 633-168                                      | 4696                       | 767-184                                      | 6692                       |
| 634-169                                      | 3090                       | 768-185                                      | 1353                       |
| 671-170                                      | 2417                       | 771-186                                      | 1153                       |
| 672-171                                      | 2840                       | 772-187                                      | 3800                       |
| 829-172                                      | 3320                       | 780-188                                      | 3740                       |
| 830-173                                      | 1833                       | 843-189                                      | 163                        |
| 831-174                                      | 416                        | 844-190                                      | 122                        |
| 792-175                                      | 126                        | 845-191                                      | 427                        |
| 793-176                                      | 2559                       | 846-192                                      | 2674                       |
| 794-177                                      | 289                        | 887-193                                      | 453                        |
| 732-178                                      | 179                        | 888-194                                      | 2996                       |
| 733-179                                      | 1329                       | 889-195                                      | 1075                       |
| 734-180                                      | 5665                       |                                              |                            |
| MEDIAN<br>p MALUE (M-W)                      | 1817                       | MEDIAN<br>p MALUE (M-W)                      | 1153                       |
| MEAN<br>ST. DEV.<br>% CV<br>p VALUE (t TEST) | 1931<br>1718<br>89<br>n=16 | MEAN<br>ST. DEV.<br>% CV<br>p VALUE (t TEST) | 1825<br>1769<br>97<br>n=15 |

FIG. 15A

10/13

## CORTEX

| 2 mg ALUM<br>100 µg AN1528                   |                            | 50 µg MPL<br>100 µg AN1528                   |                            |
|----------------------------------------------|----------------------------|----------------------------------------------|----------------------------|
| 660-083                                      | 295                        | 643-105                                      | 385                        |
| 661-084                                      | 3180                       | 644-106                                      | 2640                       |
| 662-085                                      | 2480                       | 645-107                                      | 2403                       |
| 663-086                                      | 3014                       | 654-108                                      | 1741                       |
| 664-087                                      | 5870                       | 655-109                                      | 3053                       |
| 665-088                                      | 5978                       | 656-110                                      | 5990                       |
| 693-089                                      | 1620                       | 678-111                                      | 3360                       |
| 694-090                                      | 35                         | 679-112                                      | 1230                       |
| 695-091                                      | 3400                       | 704-114                                      | 2680                       |
| 697-092                                      | 2630                       | 705-115                                      | 78                         |
| 698-093                                      | 983                        | 706-116                                      | 1290                       |
| 699-094                                      | 5327                       | 729-117                                      | 3180                       |
| 701-095                                      | 1862                       | 730-118                                      | 1833                       |
| 702-096                                      | 1849                       | 731-119                                      | 4590                       |
| 703-097                                      | 2239                       | 736-120                                      | 1112                       |
| 739-098                                      | 806                        | 737-121                                      | 1653                       |
| 740-099                                      | 5303                       | 757-122                                      | 992                        |
| 741-100                                      | 459                        | 758-123                                      | 4692                       |
| 800-103                                      | 154                        | 808-124                                      | 785                        |
| 801-104                                      | 852                        | 809-125                                      | 244                        |
|                                              |                            | 810-126                                      | 32                         |
| MEDIAN<br>p MALUE (M-W)                      | 2051                       | MEDIAN<br>p MALUE (M-W)                      | 1741                       |
| MEAN<br>ST. DEV.<br>% CV<br>p VALUE (t TEST) | 2407<br>1913<br>79<br>n=20 | MEAN<br>ST. DEV.<br>% CV<br>p VALUE (t TEST) | 2140<br>1659<br>78<br>n=21 |

FIG. 15B

11/13

## CORTEX

| 25 µg QS21<br>100 µg AN1528 |      | CEA/IFA<br>100 µg AN1792 |        |
|-----------------------------|------|--------------------------|--------|
| 615-128                     | 1257 | 539-068                  | 693    |
| 616-129                     | 361  | 640-069                  | 508    |
| 617-130                     | 1008 | 641-070                  | 440    |
| 536-131                     | 3290 | 642-071                  | 467    |
| 637-132                     | 2520 | 690-072                  | 42     |
| 638-133                     | 3880 | 691-073                  | 2491   |
| 744-134                     | 627  | 692-074                  | 121    |
| 745-135                     | 58   | 795-075                  | 137    |
| 746-136                     | 2610 | 796-076                  | 822    |
| 747-137                     | 1509 | 797-077                  | 475    |
| 769-138                     | 1788 | 748-087                  | 600    |
| 770-139                     | 988  | 749-079                  | 78     |
| 773-140                     | 1199 | 750-080                  | 1267   |
| 774-141                     | 339  | 751-081                  | 1351   |
| 775-142                     | 402  | 761-082                  | 69     |
| 776-143                     | 537  |                          |        |
| 840-144                     | 1119 |                          |        |
| 841-145                     | 194  |                          |        |
| 821-146                     | 1259 |                          |        |
| 822-147                     | 5413 |                          |        |
| 823-148                     | 2233 |                          |        |
| MEDIAN                      | 1199 | MEDIAN                   | 475    |
| p MALUE (M-W)               |      | p MALUE (M-W)            | 0.0481 |
| MEAN                        | 1552 | MEAN                     | 637    |
| ST. DEV.                    | 1364 | ST. DEV.                 | 655    |
| % CV                        | 88   | % CV                     | 103    |
| p VALUE (t TEST)            |      | p VALUE (t TEST)         | 0.0106 |
| n=21                        |      | n=15                     |        |

FIG. 15C



12/13

## CORTEX

| 5 µg THIMEROSAL/PBS<br>10 µg AN1792          |                            | 2 µg ALUM<br>100 µg AN1792                   |                                      |
|----------------------------------------------|----------------------------|----------------------------------------------|--------------------------------------|
| 635-149                                      | 1337                       | 610-001                                      | 432                                  |
| 669-150                                      | 4644                       | 611-002                                      | 1012                                 |
| 670-151                                      | 6335                       | 612-003                                      | 3607                                 |
| 673-152                                      | 3700                       | 613-004                                      | 508                                  |
| 674-153                                      | 2750                       | 620-005                                      | 465                                  |
| 676-154                                      | 1687                       | 621-006                                      | 16                                   |
| 681-156                                      | 185                        | 622-007                                      | 28                                   |
| 682-157                                      | 8031                       | 623-008                                      | 217                                  |
| 683-158                                      | 3450                       | 708-009                                      | 2738                                 |
| 754-159                                      | 157                        | 709-010                                      | 927                                  |
| 755-160                                      | 6857                       | 710-011                                      | 1609                                 |
| 756-161                                      | 482                        | 716-012                                      | 1608                                 |
| 805-162                                      | 524                        | 784-014                                      | 3890                                 |
| 806-163                                      | 397                        | 785-015                                      | 1614                                 |
| 807-164                                      | 234                        | 786-018                                      | 285                                  |
|                                              |                            | 787-017                                      | 3102                                 |
|                                              |                            | 788-018                                      | 1617                                 |
|                                              |                            | 789-019                                      | 1474                                 |
|                                              |                            | 815-020                                      | 424                                  |
|                                              |                            | 816-021                                      | 1375                                 |
|                                              |                            | 817-022                                      | 2323                                 |
| MEDIAN<br>p MALUE (M-W)                      | 1687                       | MEDIAN<br>p MALUE (M-W)                      | 1375<br>0.5000                       |
| MEAN<br>ST. DEV.<br>% CV<br>p VALUE (t TEST) | 2718<br>2685<br>99<br>n=15 | MEAN<br>ST. DEV.<br>% CV<br>p VALUE (t TEST) | 1394<br>1166<br>84<br>0.2650<br>n=21 |

FIG. 15D

13/13

## CORTEX

| 50 µg MPL<br>100 µg AN1792 |        | 25 µg QS21<br>100 µg AN1792 |        |
|----------------------------|--------|-----------------------------|--------|
| 646-023                    | 2002   | 627-045                     | 91     |
| 647-024                    | 147    | 628-046                     | 3397   |
| 648-025                    | 1304   | 631-049                     | 3702   |
| 649-026                    | 34     | 632-050                     | 1776   |
| 650-027                    | 980    | 667-052                     | 1832   |
| 724-028                    | 1282   | 668-053                     | 3023   |
| 726-030                    | 1966   | 686-054                     | 189    |
| 727-031                    | 733    | 687-055                     | 891    |
| 720-032                    | 2563   | 688-056                     | 240    |
| 721-033                    | 5563   | 689-057                     | 110    |
| 802-034                    | 113    | 712-059                     | 3311   |
| 803-035                    | 671    | 825-061                     | 1009   |
| 804-036                    | 51     | 826-082                     | 18165  |
| 811-037                    | 613    | 827-063                     | 73     |
| 812-038                    | 332    | 828-064                     | 78     |
| 813-039                    | 1454   | 837-065                     | 1051   |
| 814-040                    | 2441   | 838-066                     | 270    |
| 833-014                    | 742    | 839-067                     | 371    |
| 834-042                    | 40     |                             |        |
| 836-044                    | 807    |                             |        |
| MEDIAN                     | 774    | MEDIAN                      | 950    |
| p MALUE (M-W)              | 0.1710 | p MALUE (M-W)               | 0.4076 |
| MEAN                       | 1192   | MEAN                        | 2199   |
| ST. DEV.                   | 1299   | ST. DEV.                    | 4187   |
| % CV                       | 109    | % CV                        | 190    |
| p VALUE (t TEST)           | 0.1506 | p VALUE (t TEST)            | 0.8131 |
|                            | n=21   |                             | n=18   |

FIG. 15E

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/25386

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A61K 38/00, 38/28, 9/26, 33/06

US CL : 424/88, 92, 570, 698; 514/ 2, 4, 21

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/88, 92, 570, 698; 514/ 2, 4, 21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN: COMPOUNDS AND METHODS OF USE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                | Relevant to claim No. |
|-----------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Y         | EP 0 526 511 B1 (MCMICHAEL) 28 MAY 1997, see entire document                                                                                                                                                                      | 1-66                  |
| Y         | Chemical Abstracts, volume 120, number 8, issued 21 February 1994, Prieels et al, "Synergistic adjuvants for vaccines" page 652, column 1, abstract no. 86406t, PCT Int. Appl. WO 94 00,153, 06 January 1994, see entire abstract | 1-66                  |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

|                                                                                                                                                                         |                                                                                                                                                                                                                                                  |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| * Special categories of cited documents:                                                                                                                                | *T* later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention                                             |
| *A* document defining the general state of the art which is not considered to be of particular relevance                                                                | *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone                                                                     |
| *B* earlier document published on or after the international filing date                                                                                                | *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | *G* document member of the same patent family                                                                                                                                                                                                    |
| *O* document referring to an oral disclosure, use, exhibition or other means                                                                                            |                                                                                                                                                                                                                                                  |
| *P* document published prior to the international filing date but later than the priority date claimed                                                                  |                                                                                                                                                                                                                                                  |

Date of the actual completion of the international search

18 MARCH 1999

Date of mailing of the international search report

08 APR 1999

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

RUSSELL TRAVERS

Telephone No. (703) 308-1235



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |           |                                                                                                                                                                                                                                    |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>(51) International Patent Classification <sup>6</sup> :</b><br><b>A61K 38/17</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      | <b>A1</b> | <b>(11) International Publication Number:</b> <b>WO 99/27949</b><br><b>(43) International Publication Date:</b> 10 June 1999 (10.06.99)                                                                                            |
| <b>(21) International Application Number:</b> PCT/US98/25694<br><b>(22) International Filing Date:</b> 3 December 1998 (03.12.98)<br><br><b>(30) Priority Data:</b><br>60/067,219                      3 December 1997 (03.12.97)      US<br>60/079,697                      27 March 1998 (27.03.98)        US<br><br><b>(71) Applicant:</b> BRIGHAM AND WOMEN'S HOSPITAL<br>[US/US]; 75 Francis Street, Boston, MA 02115 (US).<br><br><b>(72) Inventors:</b> WEINER, Howard, L.; 114 Somerset Road, Brook-<br>line, MA 02146 (US). SELKOE, Dennis, J.; 166 Moss Hill<br>Road, Jamaica Plain, MA 02130 (US).<br><br><b>(74) Agents:</b> JACOBS, Seth, H. et al.; Darby & Darby P.C., 805<br>Third Avenue, New York, NY 10022-7513 (US). |           | <b>(81) Designated States:</b> AU, BR, CA, HU, IL, JP, KR, NO,<br>European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR,<br>GB, GR, IE, IT, LU, MC, NL, PT, SE).<br><br><b>Published</b><br><i>With international search report.</i> |
| <b>(54) Title:</b> METHOD OF SUPPRESSING $\beta$ -AMYLOID-RELATED CHANGES IN ALZHEIMER'S DISEASE<br><br><b>(57) Abstract</b><br><br>Methods are provided for treating or preventing Alzheimer's disease comprising mucosally administering $\beta$ -amyloid peptide or a fragment or analog thereof in an oral or other mucosal dosage form. Also disclosed herein are pharmaceutical formulation or dosage forms for use in these methods.                                                                                                                                                                                                                                                                                              |           |                                                                                                                                                                                                                                    |

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

|    |                          |    |                                          |    |                                              |    |                          |
|----|--------------------------|----|------------------------------------------|----|----------------------------------------------|----|--------------------------|
| AL | Albania                  | ES | Spain                                    | LS | Lesotho                                      | SI | Slovenia                 |
| AM | Armenia                  | FI | Finland                                  | LT | Lithuania                                    | SK | Slovakia                 |
| AT | Austria                  | FR | France                                   | LU | Luxembourg                                   | SN | Senegal                  |
| AU | Australia                | GA | Gabon                                    | LV | Latvia                                       | SZ | Swaziland                |
| AZ | Azerbaijan               | GB | United Kingdom                           | MC | Monaco                                       | TD | Chad                     |
| BA | Bosnia and Herzegovina   | GE | Georgia                                  | MD | Republic of Moldova                          | TG | Togo                     |
| BB | Barbados                 | GH | Ghana                                    | MG | Madagascar                                   | TJ | Tajikistan               |
| BE | Belgium                  | GN | Guinea                                   | MK | The former Yugoslav<br>Republic of Macedonia | TM | Turkmenistan             |
| BF | Burkina Faso             | GR | Greece                                   | ML | Mali                                         | TR | Turkey                   |
| BG | Bulgaria                 | HU | Hungary                                  | MN | Mongolia                                     | TT | Trinidad and Tobago      |
| BJ | Benin                    | IE | Ireland                                  | MR | Mauritania                                   | UA | Ukraine                  |
| BR | Brazil                   | IL | Israel                                   | MW | Malawi                                       | UG | Uganda                   |
| BY | Belarus                  | IS | Iceland                                  | MX | Mexico                                       | US | United States of America |
| CA | Canada                   | IT | Italy                                    | NE | Niger                                        | UZ | Uzbekistan               |
| CF | Central African Republic | JP | Japan                                    | NL | Netherlands                                  | VN | Viet Nam                 |
| CG | Congo                    | KE | Kenya                                    | NO | Norway                                       | YU | Yugoslavia               |
| CH | Switzerland              | KG | Kyrgyzstan                               | NZ | New Zealand                                  | ZW | Zimbabwe                 |
| CI | Côte d'Ivoire            | KP | Democratic People's<br>Republic of Korea | PL | Poland                                       |    |                          |
| CM | Cameroon                 | KR | Republic of Korea                        | PT | Portugal                                     |    |                          |
| CN | China                    | KZ | Kazakstan                                | RO | Romania                                      |    |                          |
| CU | Cuba                     | LC | Saint Lucia                              | RU | Russian Federation                           |    |                          |
| CZ | Czech Republic           | LI | Liechtenstein                            | SD | Sudan                                        |    |                          |
| DE | Germany                  | LK | Sri Lanka                                | SE | Sweden                                       |    |                          |
| DK | Denmark                  | LR | Liberia                                  | SG | Singapore                                    |    |                          |
| EE | Estonia                  |    |                                          |    |                                              |    |                          |

**METHOD OF SUPPRESSING  $\beta$ -AMYLOID-RELATED  
CHANGES IN ALZHEIMER'S DISEASE**

This application claims priority under 35 U.S.C. §119 of U.S. application serial no. 60/067,219, filed December 3, 1997 and U.S. application serial no. 60/079,697, filed March 27, 1998, which applications are hereby incorporated by reference in their entireties.

Field of the Invention

This invention relates to a treatment for Alzheimer's disease based on mucosal tolerization.

Background of the Invention

1. Alzheimer's Disease

Alzheimer's disease is the most common cause of cognitive degeneration in the elderly. Selkoe, D.J., et al., Science, **235**, 873-877 (1987). Extensive synaptic loss occurs with resulting progressive memory loss, psychological instability, decline in abstract reasoning, loss of bodily function, coma, and ultimately death. Extracellular lesions known as senile plaques develop in the brains of Alzheimer's victims, comprising insoluble aggregates of  $\beta$ -amyloid ( $A\beta$ ) protein surrounded by dystrophic neurites. Mullan, M. and Crawford, F., Trends Neurosci., **16**, 398-403 (1993).  $\beta$ -Amyloid is a hydrophobic peptide comprising 39-43 amino acid residues; it is characterized by a high tendency to form aggregates, and results from proteolytic processing of a 90-140 kDa integral membrane protein precursor known as amyloid precursor protein (APP).

While formerly it was thought that  $\beta$ -amyloid peptide itself was responsible for neurodegeneration in

Alzheimer's disease since the peptide can kill neurons in vitro, it is now known that the APP precursor is expressed in the brain cells of both healthy and Alzheimer's diseased brains. Konig, G., et al., Mol. Brain Res., **9**, 259-262

5 (1991). Hence, presence of the  $\beta$ -amyloid peptide is not by itself sufficient to account for the disease.

Attention has recently shifted to the potential involvement or participation in Alzheimer's of immunological processes such as inflammation. A large body of research  
10 evidence has accumulated showing certain parallels between immunological events and Alzheimer's disease. Alzheimer's brains display various structural features of immune-mediated brain damage including increased populations of microglial cells and astrocytes which express such  
15 inflammatory cytokines and inflammation-associated agents as interleukin-1 and  $\alpha$ 1-antichymotrypsin, and which are immunoreactive with major histocompatibility complex (MHC) class I and II. Abraham, et al., Cell, **52**, 487 (1988). Microglial cells are thought to be the functional equivalent  
20 of macrophages in the central nervous system. Stryen, et al., Exp. Neurol., **110**, 93 (1990). Other features observed in both immune-mediated and Alzheimer's brain damage include immunoreactive markers such as MHC class I and II glycoproteins, cytokines, complement receptors and  
25 regulatory factors. McGeer, P.L., et al., Neurosci. Lett., **107**, 341-344 (1989); McGeer, P.L., et al., Brain Res., **544**, 315-320 (1990); Griffin, W.S., et al., Proc. Nat'l. Acad. Sci. U.S.A., **86**, 7611-7615 (1989). Also, the inflammatory mediator interleukin-6 (IL-6) which is found in Alzheimer's  
30 brains is known to stimulate  $\alpha$ -2-macroglobulin ( $\alpha$ 2M), a highly potent human protease inhibitor produced by neurons of Alzheimer's brains, and thought to interfere with normal proteolytic processing of APP to form  $\beta$ -amyloid. Bauer, J., et al., FEBS Letts., **285**, 111-114 (1991). The physiological  
35 production of  $\beta$ -amyloid protein and mechanism of Alzheimer's disease is further described in Selkoe, Trends in Neurosciences., 16:403-409 (1993).

It is also known that  $\beta$ -amyloid peptide present in insoluble form in Alzheimer's brain plaques can bind and activate the classical complement proteins (C1q) *in vitro* in the absence of antibody proteins (e.g., immunoglobulins).

5 In contrast, the soluble phase A $\beta$  is subject to protective regulation by fluid-phase inhibitors, suggesting that it is the insoluble form of A $\beta$  that is neurotoxic and explaining the apparent harmlessness of soluble A $\beta$ . Rogers, et al., Proc. Nat'l Acad. Sci. U.S.A., **89**, 10016-10020 (1992).

10 McGeer and Rogers and their collaborators have proposed that aggregated  $\beta$ -amyloid may activate the complement cascade which leads to the generation of proteins capable of destroying neurons. Cf. Schnabel, J., New Scientist, **138**, 22 (June 19, 1993). In further support of a mechanism link  
15 between amyloid plaque deposition and immune response, it was found that microglial cells, the immune cells of the brain, can be activated with interferon- $\gamma$  (IFN- $\gamma$ ) and [1-42]A $\beta$  to produce significantly more damage to neurons than either stimulus alone. Meda, L., et al., Nature, **374**, 647-  
20 650 (1995). This finding suggests a synergistic effect between A $\beta$  and IFN- $\gamma$  in triggering the generation by microglial cells of neurodegenerative substances such as reactive nitrogen intermediates (e.g., toxic free-radicals) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). See, also Huberman, M.  
25 et al., J. Neuroimmunol., **52**:147-152, 1994 who report increased secretion in Alzheimer's patients of proinflammatory cytokines, and a correlation between cytokine levels and stage of the disease.

Epidemiological evidence also supports a nexus  
30 between immunological activity and Alzheimer's disease. Anderson, K., et al., Neurol., **45**, 1441-1445 (1995). In the Rotterdam study surveying 7,983 subjects, non-steroidal anti-inflammatory drugs were shown to have a protective effect on the risk for developing Alzheimer's disease and an  
35 ameliorating effect on the disease component of diminished cognitive function. In spite of these studies pointing to a role for the immune system in the induction of Alzheimer's



disease, no truly effective therapeutic regimes have emerged based on these research results.

Current treatments for Alzheimer's disease are generally few and inadequate. TACRINE<sup>TM</sup> (1,2,3,4-tetrahydro-9-acridinamine monohydrochloride monohydrate; also marketed  
5 as COGNEX<sup>TM</sup>) is a reversible cholinesterase inhibitor with some efficacy for treating Alzheimer's disease. Watkins, P.B., et al., J. Amer. Med. Assoc., **271**, 992 (1994).

TACRINE has been only partially effective in community-  
10 dwelling subjects with mild or moderate dementia. Weiner, M.F., Consultant, **35**, 313 (1995). Aside from its limited efficacy, there are safety concerns for its use. These include the elevation of serum aminotransferase levels which may indicate hepatocellular injury. Other dangers involve a  
15 risk of developing liver necrosis, jaundice or hepatitis which can be severe or fatal in particularly susceptible patients.

Other approaches have been attempted, such as the treatment of psychological symptoms of Alzheimer's disease  
20 using selective serotonin re-uptake inhibitors, e.g., paroxetine, fluoxetine, or trazodone, and the administration of cholinergic agonists or precursors, e.g., lecithin and nicotine but have met with similarly limited success. Pilot clinical studies suggest that nicotine may be useful to  
25 treat deficits in attention and information processing resulting from Alzheimer's disease. Sahakian, et al., Brit. J. Psych., **154**, 797 (1989). Nicotine, however, is poorly bioavailable, and therefore not useful as a clinical treatment.

30 Viewing Alzheimer's disease as a chronic inflammatory state, McGeer and Rogers have proposed using anti-inflammatory agents such as indomethacin, a drug which curbs inflammation by blocking the action of cyclooxygenase, to treat the disease. McGeer, P.L., et al., U.S. Patent No.  
35 5,192,753, issued March 9, 1993. In a study of 44 subjects with early-stage dementia, it was found that the group receiving the drug indomethacin did not deteriorate in mental function during the study. Schnabel, J., New

Scientist, *supra*. However, various side effects of indomethacin, including gastro-intestinal effects, make its clinical use problematic.

Garvey, *et al.*, U.S. Patent No. 5,409,946, issued April 25, 1995, disclose certain isoxazole, isothiazole and pyrazole compounds which allegedly enhance cognitive function, but do not appear to treat Alzheimer's disease directly.

In general, all known methods of treating Alzheimer's disease have met with no significant success. A clear need therefore exists for improved methods of treating the disease.

## 2. Oral and, More Generally, Mucosal Tolerization

A mucosal tolerization approach to treating T-cell mediated autoimmune diseases is discussed extensively in PCT International Application No. PCT/US93/01705. This method involves the oral or mucosal administration of antigens specific to the tissue under autoimmune attack which may or may not be themselves a target of the autoimmune attack ("bystander antigens"). Oral or mucosal administration of these "bystander antigens" causes active suppression, i.e. the induction of regulatory (suppressor) T-cells in the gut-associated lymphoid tissue (GALT), or, in the case of by-inhalation administration, bronchial-associated lymphoid tissue (BALT). See, also Miller, A., *et al.*, J. Exp. Med., **174**, 791-798 (1991). These regulatory suppressor T-cells are released in the blood or lymphatic tissue and then migrate to the organ or tissue afflicted with the autoimmune disease where they suppress autoimmune attack mounted against the afflicted organ or tissue. The T-cells elicited by the bystander antigen (which recognize at least one epitope of the bystander antigen used to elicit them) are targeted to the locus of autoimmune attack where they mediate the local release of one or more immunomodulatory cytokines, such as transforming growth factor beta (TGF- $\beta$ ) interleukin-4 (IL-4) or interleukin-10 (IL-10) which down-regulate immune response.

Of these, TGF- $\beta$  is an antigen-nonspecific immunosuppressive factor in that it suppresses all immune attack phenomena in the vicinity of its release regardless of the antigen that triggers these phenomena. (However, because oral tolerization with a bystander antigen causes the release of immunoregulatory substances only in the vicinity of autoimmune attack, no systemic immunosuppression ensues.)

IL-4 and IL-10 are also antigen-nonspecific immunoregulatory cytokines. IL-4 in particular enhances Th2 response, *i.e.*, it acts on T-cell precursors and causes them to differentiate preferentially into Th2 cells. IL-4 also indirectly inhibits Th1 exacerbation. IL-10 is a direct inhibitor of Th1 responses.

After mucosally tolerizing mammals afflicted with an autoimmune disease, increased levels of TGF- $\beta$ , IL-4 and IL-10 were observed in the gut associated lymphoid tissue (Chen, Y., *et al.*, Science, **265**, 1237-1240 (1994)) and a concomitant decrease in cytokines associated with immune system (and particularly T-cell activation), such as IL-2, IFN- $\gamma$ , etc. Downregulation of inflammatory cytokines and upregulation of TGF- $\beta$ , and IL-4 were observed in the brains of EAE rats following mucosal tolerization of those animals (Khoury *et al.*, J. Exp. Med., **176**, 1355-1364 (1992)).

Naive T-cells differentiate into distinct populations defined by their cytokine secretion pattern and regulatory function. Th1 cells secrete predominantly interleukin-2 (IL-2) and IFN- $\gamma$  and are involved in classic delayed-type hypersensitivity reactions, while Th2 cells, which secrete predominantly IL-4 and IL-10, induce selected immunoglobulin secretion and can down-regulate Th1-mediated immune responses Mossmann, T. R., *et al.*, J. Immunol., **136**, 2348-57 (1986); Romagnan, S., Annu. Rev. Immunol., **12**, 227-57 (1994). Thus, cytokines secreted by T-cells after activation qualitatively influence the nature of the immune response. Recent reports have suggested that the dominant factors determining the maturation of naive T-cells into either predominantly Th1 or Th2 cells are cytokines,

particularly IL-2 and IFN- $\gamma$  for Th1 and IL-4 for Th2. Seder, R. A., et al., Annu. Rev. Immunol., **12**, 635-673 (1994).

Other factors include the dose and type of antigen, the type of antigen-presenting cell (APC), and co-stimulatory molecules involved in activation. Once differentiated into Th1 or Th2 type, T-cells were formerly thought to be committed to the production of a given set of lymphokines upon restimulation. However, it has been recently discovered that certain MBP-specific, CD4<sup>+</sup> T-cells, isolated from the GALT of SJL mice orally tolerized with myelin basic protein and MS patients orally tolerized with myelin and structurally identical to Th1 disease-inducing clones in terms of their T-cell receptor usage, MHC restriction and epitope recognition, suppressed rather than induced disease and may represent a novel T-cell subset (Th3) with both mucosal T-helper function and down-regulatory properties vis-a-vis Th1 and other immune cells.

T-cell secretion of certain cytokines is also implicated in the induction and regulation of autoimmune inflammatory disease, which is mediated by activated autoreactive T-cells that recognize self-tissue-specific antigen in the context of MHC class II molecules on APCs. In experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis, it is believed that T-cell secretion of IL-2, IFN- $\gamma$ , and TNF mediates inflammation and tissue damage, while the secretion of IL-4, IL-10, and TGF- $\beta$ 1 by myelin basic protein (MBP)-reactive T-cells is associated with potent suppressor activity and down-regulation of central nervous system inflammation. Miller, A., et al., Proc. Nat'l Acad. Sci. U.S.A., *supra* (1992). The induction of anti-inflammatory cytokines, including TGF- $\beta$ 1, appears to be of particular importance in regulating EAE. For example, anti-TGF- $\beta$ 1 monoclonal antibodies inhibit the suppressor effect of regulatory MBP-reactive T-cells. Chen, Y., et al., Science, *supra* (1994). Thus, autoreactive T-cells are not necessarily pathologic and can function to down-regulate immune responses associated with tissue inflammation locally. Similar findings have been made in humans

afflicted with multiple sclerosis. TNF has been isolated from central nervous system plaques of MS patients, IFN- $\gamma$  exacerbates attacks, and TGF- $\beta$  and/or IL-4 and/or IL-10 is secreted by T-cells of MS patients tolerized with myelin.

5 Objects of the Invention

It is an object of the present invention to provide novel immunomodulatory methods for treating individuals suffering from Alzheimer's disease and for preventing the disease in individuals at risk for  
10 contracting the disease.

Another object of the present invention is to provide compositions and pharmaceutical formulations useful for preventing the induction of Alzheimer's disease in animal models and for treating mammals suffering from the  
15 disease.

It is a further object of this invention to devise methods and compositions for suppressing certain cytokine responses associated with Alzheimer's disease in an individual by a mucosal mode of administration, and for  
20 enhancing certain other cytokine responses associated with the suppression of inflammatory responses associated with the disease.

It is another object of the invention to prevent deposition of  $\beta$ -amyloid.

25 These and other objects of the present invention will be apparent to those of ordinary skill in the art in light of the following.

Summary of the Invention

It has been unexpectedly discovered by the present  
30 inventors that mucosal administration of an antigen associated with  $\beta$ -amyloid plaques (such as  $\beta$ -amyloid peptide and fragments thereof) is an effective treatment for disease associated with said plaques. In one aspect, the invention is directed to a method for treating Alzheimer's disease in  
35 a mammal in need of such treatment, comprising orally (or more generally, mucosally) administering to the mammal an effective amount of an agent comprising whole  $\beta$ -amyloid

peptide or an effective fragment of  $\beta$ -amyloid peptide, particularly fragments recognized by T-cells of the host (including, without limitation, the peptides [1-40]A $\beta$ , [1-42]A $\beta$  and [25-35]A $\beta$ ). Preferably, the administration is continued for a period of time sufficient to achieve at least one of the following:

- (i) reduction in the level of one or more proinflammatory Th1 cytokines;
- (ii) increase in the level of one ore more anti-inflammatory Th2 or Th3 cytokines;
- (iii) amelioration, retardation or suppression of at least one clinical or histological symptom of Alzheimer's disease; or
- (iv) suppression of  $\beta$ -amyloid deposition.

Mucosally administered  $\beta$ -amyloid peptide according to the invention can initiate immunological responses in a subject which prevent, retard or arrest neuron cell destruction and/or prevent deposition of  $\beta$ -amyloid in neurons.

The present invention also relates to formulations adapted for mucosal administration, and/or delivery systems adapted for mucosal administration, comprising an antigen associated with Alzheimer's brain plaques (such as  $\beta$  amyloid peptide or a fragment thereof) and useful in the treatment of Alzheimer's disease. These and other embodiments of the subject invention will be described in the detailed description.

In another aspect of the invention,  $\beta$ -amyloid peptide (or an effective fragment or analog thereof) is mucosally administered to treat amyloid plaque formation associated with Down's syndrome, Hereditary Cerebral Hemorrhage with Amyloidosis-Dutch Type (HCHWA-D), sporadic and familial  $\beta$ -amyloid angiopathy, or other diseases involving cerebral accumulation of  $\beta$ -amyloid.

#### Brief Description of the Drawings

Figure 1A illustrates proliferation (expressed as Stimulation Index (S.I.)) of T-cells from spleens of control and experimental SWISSXBDF1 mice either fed (75  $\mu$ g/feeding x

5) or nasally treated (25  $\mu$ g/application x 3) with [1-40] $\beta$ -amyloid peptide and immunized two days after the last treatment with 100  $\mu$ g [1-40] $\beta$ -amyloid peptide in 50  $\mu$ g CFA per mouse. Proliferation was determined ten days after immunization.

Figure 1B illustrates proliferation (expressed as S.I.) of popliteal lymph node T-cells from control and experimental SWISSXBDF1 mice and mice fed or nasally administered [1-40] $\beta$ -amyloid peptide determined ten days after being immunized in the foot pad with 100  $\mu$ g [1-40] $\beta$ -amyloid peptide in 50  $\mu$ g CFA per mouse. Control mice (designated "non") were only immunized.

Figure 2A illustrates proliferation (expressed as  $\Delta$ CPM relative to nonimmunized mice) of T-cells from popliteal lymph nodes from SJL/J, DBA/1, C57BL/6, BALB/C and C3H/EB mice immunized with [1-40] $\beta$ -amyloid peptide but not fed any peptide.

Figure 2B illustrates proliferation (expressed as  $\Delta$ CPM as in Fig. 2A) of spleen T-cells from SJL/J, DBA/1, C57BL/6, BALB/C and C3H/EB mice immunized with [1-40] $\beta$ -amyloid peptide but not fed any peptide.

Figure 3A illustrates the secretion (expressed as  $\Delta$ pg/ml relative to nonimmunized controls) of cytokines IL-2, IFN- $\gamma$ , IL-4, IL-10 and TGF- $\beta$  in popliteal lymph node T-cells from SJL/J, DBA/1, C57BL/6, BALB/C and C3H/EB mice immunized with [1-40] $\beta$ -amyloid peptide but not fed any peptide.

Figure 3B illustrates the secretion (expressed in  $\Delta$ pg/ml as in Fig. 3A) of cytokines IL-2, IFN- $\gamma$ , IL-4, IL-10 and TGF- $\beta$  in T-cells isolated from spleens of SJL/J, DBA/1, C57BL/6, BALB/C and C3H/EB mice immunized with [1-40] $\beta$ -amyloid peptide but not fed any peptide.

Figure 4A illustrates the secretion (expressed as in Fig. 3A) of cytokine IL-10 in T-cells from popliteal

lymph nodes of C3H/EB mice either fed (5, 50 or 500  $\mu$ g/feeding for 5 days) or nasally administered (5 or 50  $\mu$ g/application for 3 days) [1-40] $\beta$ -amyloid peptide prior to immunization (2 days after the last feeding or nasal application) with 100  $\mu$ g [1-40] $\beta$ -amyloid peptide in 50  $\mu$ g CFA.

Figure 4B illustrates the secretion (expressed as in Fig. 3A) of cytokine IL-10 in T-cells from spleens of C3H/EB mice either fed (5, 50 or 500  $\mu$ g/feeding for 5 days) or nasally administered (5 or 50  $\mu$ g/application for 3 days) [1-40] $\beta$ -amyloid peptide prior to immunization (2 days after the last feeding or nasal application) with 100  $\mu$ g [1-40] $\beta$ -amyloid peptide in 50  $\mu$ g CFA.

Figure 5A illustrates the secretion (expressed as in Fig. 3A) of cytokine IL-2 in popliteal lymph node T-cells from C3H/EB mice either fed (5, 50 or 500  $\mu$ g/feeding for 5 days) or nasally administered (5 or 50  $\mu$ g/application for 3 days) [1-40] $\beta$ -amyloid peptide prior to immunization (2 days after the last feeding or nasal application) with 100  $\mu$ g [1-40] $\beta$ -amyloid peptide in 50  $\mu$ g CFA.

Figure 5B illustrates the secretion (expressed as in Fig. 3A) of TGF- $\beta$  cytokine IL-2 in T-cells from spleens of C3H/EB mice either fed (5, 50 or 500  $\mu$ g/feeding for 5 days) or nasally administered (5 or 50  $\mu$ g/application for 3 days) [1-40] $\beta$ -amyloid peptide prior to immunization (2 days after the last feeding or nasal application) with 100  $\mu$ g [1-40] $\beta$ -amyloid peptide in 50  $\mu$ g CFA.

Figure 6A illustrates the secretion (expressed as in Fig. 3A) of TGF- $\beta$  in popliteal lymph node T-cells from C3H/EB mice either fed (5, 50 or 500  $\mu$ g/feeding for 5 days) or nasally administered (5 or 50  $\mu$ g/application for 3 days) [1-40] $\beta$ -amyloid peptide prior to immunization (2 days after the last feeding or nasal application) with 100  $\mu$ g [1-40] $\beta$ -amyloid peptide in 50  $\mu$ g CFA.



Figure 6B illustrates the secretion (expressed as in Fig. 3A) of TGF- $\beta$  in T-cells from spleens of C3H/EB mice either fed (5, 50 or 500  $\mu$ g/feeding for 5 days) or nasally administered (5 or 50  $\mu$ g/application for 3 days) [1-40] $\beta$ -amyloid peptide prior to immunization (2 days after the last feeding or nasal application) with 100  $\mu$ g [1-40] $\beta$ -amyloid peptide in 50  $\mu$ g CFA.

Figure 7A illustrates the secretion (expressed as in Fig. 3A) of IFN- $\gamma$  from popliteal lymph node T-cells from SWISSXBDF1 mice fed 0 or 75 mg [1-40] $\beta$ -amyloid peptide 5 times or nasally administered 25 mg of the same peptide 3 times.

Figure 7B illustrates the secretion (expressed as in Fig. 3A) of IL-2, IL-10, IL-4 and TGF- $\beta$  from T-cells as described for Figure 7A.

Figure 8A illustrates the secretion (expressed as in Fig. 3A) of IFN- $\gamma$  from T-cells as described in Fig. 6B.

Figure 8B illustrates the secretion of IL-2, IL-4, IL-10 and TGF- $\beta$  from T-cells as described in Fig. 6B.

Figures 9A and 9B list results from the assessment of plaque burden, cytokine secretion, and inflammatory markers based on examination of brain sections obtained from PDAPP mice that were orally or nasally administered [1-40] $\beta$ -amyloid.

Figure 10 shows P values calculated using the 1-tailed Mann-Whitney U-test for various group comparisons among the PDAPP mice that were orally or nasally administered [1-40] $\beta$ -amyloid.

Figure 11 shows results of immunoassays conducted on mouse antisera obtained from PDAPP mice to determine the presence of various subtypes of antibodies to  $\beta$ -amyloid.

Figure 12 is a bar graph showing results of *in vitro* experiments in which PLN from PDAPP mice that were immunized with [1-40]  $\beta$ -amyloid peptide prior to sacrifice were tested for production of IL-2.

5

Figure 13 is a bar graph showing results of *in vitro* experiments in which PLN from PDAPP mice that were immunized with [1-40]  $\beta$ -amyloid peptide prior to sacrifice were tested for proliferation in the presence of [1-40]  $\beta$ -amyloid peptide.

10

Figure 14 is a bar graph showing results of *in vitro* experiments in which PLN from PDAPP mice that were immunized with [1-40]  $\beta$ -amyloid peptide prior to sacrifice were tested for production of IFN- $\gamma$ .

15

Figure 15 is a bar graph showing results of *in vitro* experiments in which PLN from PDAPP mice that were immunized with [1-40]  $\beta$ -amyloid peptide prior to sacrifice were tested for production of IL-6.

20

Figure 16 is a bar graph showing results of *in vitro* experiments in which PLN from PDAPP mice that were immunized with [1-40]  $\beta$ -amyloid peptide prior to sacrifice were tested for production of IL-10.

25

Figure 17 is a bar graph showing results of *in vitro* experiments in which PLN from PDAPP mice that were immunized with [1-40]  $\beta$ -amyloid peptide prior to sacrifice were tested for production of TGF- $\beta$ .

30

Figure 18 shows results from an experiment in which [1-40]  $\beta$ -amyloid was nasally administered to (B6D2)F1 mice in conjunction with nasal administration of IL-4 or IL-10, and prior to immunization of the mice with [1-40]  $\beta$ -amyloid. The Figure shows proliferation *in vitro* of spleen

cells from the mice in the presence of the [1-40]  $\beta$ -amyloid peptide.

5        Figure 19 shows results from an experiment in which [1-40]  $\beta$ -amyloid was nasally administered to (B6D2)F1 mice in conjunction with nasal administration of IL-4 or IL-10, and prior to immunization of the mice with [1-40]  $\beta$ -amyloid. The Figure shows production of IFN- $\gamma$  *in vitro* in spleen cells from the mice in the presence of the [1-40]  $\beta$ -amyloid peptide.  
10

Figure 20 shows results from an experiment in which [1-40]  $\beta$ -amyloid was nasally administered to (B6D2)F1 mice in conjunction with nasal administration of IL-4 or IL-10, and prior to immunization of the mice with [1-40]  $\beta$ -amyloid. The Figure shows production of IL-6 *in vitro* in spleen cells from the mice in the presence of the [1-40]  $\beta$ -amyloid peptide.  
15

Figure 21 shows results from an experiment in which [1-40]  $\beta$ -amyloid was nasally administered to (B6D2)F1 mice in conjunction with nasal administration of IL-4 or IL-10, and prior to immunization of the mice with [1-40]  $\beta$ -amyloid. The Figure shows production of IL-10 *in vitro* in spleen cells from the mice in the presence of the [1-40]  $\beta$ -amyloid peptide.  
20

25        Figure 22 shows results from an experiment in which [1-40]  $\beta$ -amyloid was nasally administered to (B6D2)F1 mice in conjunction with nasal administration of IL-4 or IL-10, and prior to immunization of the mice with [1-40]  $\beta$ -amyloid. The Figure shows production of TGF- $\beta$  *in vitro* in spleen cells from the mice in the presence of the [1-40]  $\beta$ -amyloid peptide.  
30

Detailed Description of the Invention

All publications and patent applications listed herein are hereby incorporated by reference.

The following terms as used in this disclosure have the meanings ascribed to them below.

5 "Mammal" is defined herein as any warm-blooded higher vertebrate organism (including a human) having an immune system and being susceptible to Alzheimer's disease or an induced or spontaneous animal model thereof.

10 "β-Amyloid peptide" is intended to include a peptide fragment of between about 10 to 43 (preferably 13-43) amino acid residues, the sequence of which is derived from β-amyloid precursor protein, prepared either by chemical synthetic methods known in the peptide art, including enzymatic methods or biosynthetic methods such as  
15 by isolation of peptide fragments from any source, including native tissue cultures, or by recombinant genetic engineering methods. Nonlimiting examples of β-amyloid peptide fragments encompassed by the present method and formulations include [1-40]Aβ, [1-42]Aβ, [25-35]Aβ, and  
20 analogs thereof containing substantially similar sequences and homologous, non-peptidic or isosteric replacements for selected residues. Effective peptide fragments include those having the property of suppressing a proinflammatory Th1 cytokine, decreasing the quantity of an inflammatory  
25 marker; increasing the quantity of an anti-inflammatory Th2 or Th3 cytokine, or suppressing β-amyloid deposition. Homologous replacements entail amino acid residues of related structure and differing only in the number of methylene groups present in the residue. Non-peptidic  
30 replacements include organic structures with conformational properties similar to those natural amino acid residues. Isosteric replacements refer to residues with either single atom substitutions (e.g., S for O in the aromatic hydroxyl in tyrosine) or substitutions of one moiety for another  
35 occupying a similar volume of space (e.g., an amino group for a methyl group). The useful β-amyloid peptides administered according to the invention include, among others, those having the sequences of human, bovine, and mouse β-amyloid. It is preferred to administer to humans a

purified A $\beta$  peptide having the amino acid sequence of the human or bovine A $\beta$  peptide [1-43] or a fragment thereof recognized by the T-cells of the host that suppress autoimmune reaction associated with Alzheimer's disease.

5 The peptide can form part of a longer amino acid sequence as long as the T-cell recognition site is not disturbed.

"Abatement", "suppression" or "reduction" of an immune response or reaction includes partial reduction or amelioration of one or more symptoms of the attack or  
10 reaction, e.g., reduction in number of activated T-cells or in number of antibodies or in the levels of at least one proinflammatory cytokine (e.g.  $\gamma$ -IFN, IL-2, IL-6, or TNF) or an increase in the levels of at least one anti-inflammatory cytokine, such as TGF- $\beta$ , IL-4, IL-10.

15 "Treatment" of Alzheimer's disease is intended to include, although not be limited to, one or more of the following:

(i) alteration of the profile of one or more cytokines or inflammatory markers in an Alzheimer's patient  
20 or animal model so that it conforms to or approaches the cytokine profile of a sex-and-age-matched subject without cognitive impairment;

(ii) a delay in the progress of Alzheimer's related dementia as evaluated by at least one of cognitive  
25 impairment and other NINCDS-ADRFA criteria (McKhan et al. 1984) including without limitation blood count, blood chemistry, Vitamin B12, Folic Acid, VDRL, thyroid function, electroencephalogram, computed tomography, or criteria according to the Record for Independent Living.

30 (iii) an amelioration of dementia as evaluated by one or more of the symptoms, factors and criteria detailed in item (ii) of this definition, or by observation by a physician specializing in Alzheimer's disease, or

(iv) a decrease in the amount of  $\beta$ -amyloid  
35 associated plaque than would otherwise have been observed absent the treatment.

Mucosal tolerance according to the invention is an advantageous method for treating Alzheimer's disease, dysfunction for at least the following reasons:

(1) Absence of toxicity. For example, no toxicity has been observed in clinical trials or animal experiments involving oral or other mucosal administration of other protein antigens, such as bovine myelin (which contains MBP and PLP) to humans afflicted with multiple sclerosis, or oral or by-inhalation administration of chicken Type II collagen to humans or rodents afflicted with rheumatoid arthritis (or a corresponding animal model disease); or oral administration of bovine S-antigen to humans afflicted with uveoretinitis; or oral administration of insulin to healthy volunteers.

(2) Convenience of therapy. Mucosal administration is more convenient than parenteral, or other forms, of administration.

The present inventors carried out a series of experiments to discover whether mucosal tolerization techniques causes suppression of  $\beta$ -amyloid-related inflammation characteristic of Alzheimer's disease. The use of the mucosal route to induce tolerance against an autoimmune response was found effective in an animal model to suppress undesirable immune responses associated with Alzheimer's disease, and to reduce histological symptoms of the disease in an animal model. Antigen feeding was implemented in test animals as a means of generating peripheral tolerance, and was successful in tolerizing Th1 type responses, while increasing the response of Th2 and Th3 lymphocytes, including increased anti-inflammatory cytokine responses as well as decreased popliteal lymph node proliferation in C3H/eb and DBA/1 mice. In a mouse model of Alzheimer's disease that simulates  $\beta$ -amyloid deposition, the PDAPP model, anti-inflammatory Th1 type responses were suppressed by mucosal administration of  $\beta$ -amyloid.

The present method of treating Alzheimer's disease based on the mucosal administration results, in one embodiment, in regulation of the specific immune response thought to be associated with the disease. While not wishing to be bound by theory, the present inventors believe that suppression of this response is effected primarily by an active suppression mechanism, i.e., the elicitation of T-

cells characterized by an anti-inflammatory cytokine profile. Also, as discussed below, the method of the invention has been associated with induction of antibodies of  $\beta$ -amyloid which, without wishing to be bound by any  
5 theory of the invention, may have a role in the beneficial results observed.

As shown in the results described below, the present method can also result in suppression of the deposition of  $\beta$ -amyloid. The experiments described in the  
10 Examples show that  $\beta$ -amyloid deposition was suppressed in a mouse Alzheimer's disease model.

"Mucosal" administration includes oral, enteral, intragastric, nasal, buccal or intrapulmonary administration, and more generally any method of  
15 administration (e.g. by inhalation) of an active ingredient that brings the ingredient in contact with the immune system of the treated subject at the mucosa-associated lymphoid tissue, (MALT), including that of the gut, nasal, buccal, bronchial or pulmonary mucosa. In one embodiment of the  
20 invention, an oral composition administered according to the invention is directed primarily to the gastric mucosa; this is accomplished, for example, in a dosage form that does not substantially administer the active ingredient to the oral mucosa. Also, in experiments described below,  
25 administration by inhalation has been found to be particularly effective.

In one embodiment, the present invention provides a method for treating a mammal suffering from (or at risk for developing) Alzheimer's disease comprising mucosally  
30 administering to the mammal an effective amount of a composition comprising a  $\beta$ -amyloid peptide, e.g. [1-40] $\beta$ -amyloid, [1-42] $\beta$ -amyloid, [25-35] $\beta$ -amyloid or other  $\beta$ -amyloid peptide greater than about 10 amino acids in length. In one embodiment, the  $\beta$ -amyloid peptide sequence is greater  
35 than 15 amino acids in length, and in another greater than 20 amino acids in length. Administration is preferably continued for a period of time sufficient to achieve a change in one of the parameters described above. In a

preferred embodiment, the present invention provides the method as described wherein the mammal is a human.

The present invention also provides a pharmaceutical formulation for administration to a mammal suffering from Alzheimer's disease comprising an oral or other mucosal dosage form and delivery system containing an effective amount of a composition comprising a  $\beta$ -amyloid peptide that is sufficient to achieve at achieve one of the above-described measures of treatment.

In one embodiment, the present invention provides the above-described pharmaceutical formulation wherein the oral dosage form is a solid dosage form selected from the group consisting of a tablet, a capsule and a caplet. As noted above, a preferred embodiment of the oral dosage form of the invention delivers the composition comprising the  $\beta$ -amyloid peptide primarily to the gastric mucosa. Such an embodiment includes a tablet, capsule, caplet or other dosage form that avoids substantial dissolution in the mouth, e.g., where less than 10%, preferably less than 5%, of the active ingredient in the dosage form is released in the mouth. In another embodiment, the present invention provides the pharmaceutical formulation as described above wherein the oral dosage form comprises an aqueous suspension solution of  $\beta$ -amyloid peptide. In additional embodiments, the present invention provides the pharmaceutical formulation as above-described further comprising a pharmaceutically acceptable carrier or diluent.

The present invention also provides a pharmaceutical formulation for administration to a mammal suffering from Alzheimer's disease comprising a dosage form according to the invention adapted for nasal or bronchial administration. In one preferred embodiment, the present invention provides the pharmaceutical formulation in aerosol or spray form to be delivered by inhalation as described above further comprising a pharmaceutically acceptable carrier or diluent. The formulation can, for example, be administered in a nebulizer or inhaler. Such dosage forms are used to achieve the particularly beneficial results that



have been determined by the inventors to be associated with administration by inhalation, e.g., by nasal administration.

#### Antigens that May be Used to Induce Tolerance

Suitable antigens for use in the invention include  
5 antigens specific for Alzheimer's disease, i.e. antigens  
which are recognized by immune T-cells of a human or animal  
host. Non-limiting examples include synthetic or  
biosynthetic  $\beta$ -amyloid peptide, including peptide fragments  
thereof containing between 10 and 43 residues, preferably  
10 between 13 and 43 residues. Examples of such antigens  
include the  $\beta$ -amyloid peptides [1-40]A $\beta$ , [1-42]A $\beta$  and [25-  
35]A $\beta$ . Encompassed within the scope of the present  
invention are various related substances sharing at least  
one such T-cell recognition site with  $\beta$ -amyloid peptide.  
15 Such substances include analogs of  $\beta$ -amyloid peptide which  
vary in length or composition, but containing substantially  
similar sequences (i.e., possessing significant levels,  
e.g., between about 40% and about 95%, of primary sequence  
homology). Such substances also include peptidic constructs  
20 of  $\beta$ -amyloid peptide (or aforementioned) analogs thereof and  
one or more flanking amino acid sequence found in native  $\beta$ -  
amyloid protein or of extra  $\beta$ -amyloid origin. The invention  
includes administration of amyloid precursor protein, and of  
homologs of that protein; examples are described in US  
25 Patent Nos. 5,525,714, 5,441,931, and 5,436,153. Amyloid  
precursor protein is also described in US Patent Nos.  
5,318,958 and 5,218,100. cDNA clones encoding amyloid are  
also described in US Patent No. 4,912,206. The invention  
also encompasses peptides having conservative amino acid  
30 substitutions with respect to naturally occurring  $\beta$ -amyloid  
peptides, e.g., having one or more, preferably less than  
five, conservative substitutions with respect to the human,  
bovine, or mouse sequences. Methods for assessing the  
effectiveness of such  $\beta$ -amyloid peptides,  $\beta$ -amyloid peptide  
35 analogs and constructs sharing at least one T-cell  
recognition site with a  $\beta$ -amyloid peptide are well-known in  
the art. For example, the overlapping peptide method can be  
used to test various fragments (having at least 10 and

preferably 20 amino acid residues) of  $\beta$ -amyloid peptide A $\beta$  [1-39] (or A $\beta$  [1-43] as the case may be) by placing them in contact with a preparation containing immune T-cells isolated from a host suffering from Alzheimer's disease and assessing the proliferation of such T-cells. Additionally, the cytokine patterns of such T-cells can be analyzed using the methodology provided below in the examples to further refine choice of a peptide. In one embodiment, the peptide is one which causes T-cells secreting anti-inflammatory cytokines to proliferate. In another embodiment, the peptide is one which causes suppression of one or more inflammatory responses, whether or not it causes T-cells secreting anti-inflammatory cytokines to proliferate or otherwise increases production of such anti-inflammatory cytokines. Peptides are also encompassed that suppress  $\beta$ -amyloid deposition, as shown, e.g., in the mouse model described in the Examples. In another embodiment, peptides are encompassed which cause an increase in the quantity of anti- $\beta$  amyloid antibodies present in sera, e.g., in the sera of a human or non-human host suffering from, or predisposed to, Alzheimer's disease or a disease model of Alzheimer's disease. Peptides are also encompassed that have the sequence of a  $\beta$ -amyloid peptide, but including one, two, or three altered contact points for MHC or T-cell receptor, as determined by methods known in art using cells obtained from Alzheimer's patients.

#### Formulations for Mucosal Administration

Administration of more than one antigen is possible, and may be desirable (e.g. when the patient's immune T-cells recognize more than one antigen).

Suitable formulations according to the invention include formulations adapted for oral, enteral, buccal, nasal, bronchial or intrapulmonary administration. The preparation of such formulations is well within the skill of the art. It is preferred that the antigens employed be of synthetic provenance and not isolated from biological sources to avoid the risk of infection (notably, but not exclusively, to avoid transmission of agent responsible for

the Creutzfeld-Jacob disease). Additionally, it is preferred that the formulation not contain adsorption promoting agents or ingredients that protect against proteolytic degradation. As further described below, however, the antigens can be combined with one or more enhancing agents, such as IL-4 or IL-10, to increase the effectiveness of the formulation.

Suitable oral formulations for use in tolerization of T-cell mediated immune responses according to the present invention can be in any suitable orally administrable form, for example, a pill, a liquid, or a capsule or caplet containing an effective amount of antigen. Each oral formulation may additionally comprise inert constituents including pharmaceutically acceptable carriers, diluents, fillers, disintegrants, flavorings, stabilizers, preservatives, solubilizing or emulsifying agents and salts as is well-known in the art. For example, tablets may be formulated in accordance with conventional procedures employing solid carriers and other excipients well-known in the art. Capsules may be made from any pharmaceutically acceptable materials, such as gelatine or cellulose derivatives. Nonlimiting examples of solid carriers include starch, sugar, bentonite, silica and other commonly used inert ingredients. Diluents for liquid oral formulations can include *inter alia* saline, syrup, dextrose and water.

The antigens used in the present invention can also be made up in liquid formulations or dosage forms such as, for example, suspensions or solutions in a physiologically acceptable aqueous liquid medium. Such liquid media include water, or suitable beverages, such as fruit juice or tea which will be convenient for the patient to sip at spaced apart intervals throughout the day. When given orally in liquid formulations the antigen may be dissolved or suspended in a physiologically acceptable liquid medium, and for this purpose the antigen may be solubilized by manipulation of its molecule (e.g., hydrolysis, partial hydrolysis or trypsinization) or adjustment of the pH within physiologically acceptable limits (e.g. 3.5 to 8). Alternatively, the antigen may be

reduced to micronized form and suspended in a physiologically acceptable liquid medium, or in a solution.

Sustained release oral delivery systems are also contemplated and are preferred. Nonlimiting examples of sustained release oral dosage forms include those described in U.S. Patent No. 4,704,295, issued November 3, 1987; U.S. Patent No. 4,556,552, issued December 3, 1985; U.S. Patent No. 4,309,404, issued January 5, 1982; U.S. Patent No. 4,309,406, issued January 5, 1982; U.S. Patent No. 5,405,619, issued April 10, 1995; PCT International Application WO 85/02092, published May 23, 1985; U.S. Patent No. 5,416,071, issued May 16, 1995; No. 5,371,109 issued December 6, 1994; U.S. Patent No. 5,356,635, issued Oct. 18, 1994; U.S. Patent No. 5,236,704, issued August 17, 1993; U.S. Patent No. 5,151,272, issued September 29, 1992; U.S. Patent No. 4,985,253, issued January 15, 1991; U.S. Patent No. 4,895,724, issued January 23, 1990; and U.S. Patent No. 4,675,189, issued June 23, 1987.

Sustained release oral dosage forms coated with bioadhesives can also be used. Examples are compositions disclosed in EP 516,141; U.S. Patent No. 4,226,848, Oct. 1980; U.S. Patent No. 4,713,243; U.S. Patent No. 4,940,587; PCT International Application WO 85/02092; EPO 0 205 282; Smart, et al., J. Pharm. Pharmacol., **36**, 295-99 (1984); Sala, et al., Proceed. Intem. Symp. Control. Rel. Bioact. Mater., **16**, 420-21 (1989); Hunter, et al., International Journal of Pharmaceutics, **17**, 59-64 (1983); "Bioadhesion - Possibilities and Future Trends, Kellaway," Course No. 470, May 22-24, 1989.

Commercially available sustained release formulations and devices include those marketed by ALZA Corporation, Palo Alto, CA, under tradenames ALZET, INFUSET, IVOS, OROS, OSMET, or described in one or more U.S. Patents: No. 5,284,660, issued Feb. 9, 1994; No. 5,141,750, issued Aug. 25, 1992; No. 5,110,597, issued May 5, 1992; No. 4,917,895, issued April 17, 1990; No. 4,837,027, issued June 6, 1989; No. 3,993,073, issued Nov. 23, 1976; No. 3,948,262, issued April 6, 1976; No. 3,944,064, issued March 16, 1976;

and No. 3,699,963; International Applications PCT/US93/10077 and PCT/US93/11660; and European Published Applications EP 259013 and EP 354742.

5 Sustained release compositions and devices are suitable for use in the present invention because they serve to prolong contact between the antigen and the gut-associated lymphoid tissue (GALT) and thus prolong contact between the antigen and the immune system. In addition, sustained release compositions obviate the need for discrete  
10 multi-dose administration of the antigen and permit the required amount of antigen to be delivered to GALT in one or two daily doses. This is anticipated to improve patient compliance.

Orally administrable pharmaceutical formulations  
15 containing one or more antigens such as  $\beta$ -amyloid peptides are prepared and administered to mammals who have manifested symptoms of Alzheimer's disease. Additionally, subjects who are at risk for developing Alzheimer's disease, i.e., have a genetic predisposition to developing the disease, as  
20 determined through suitable means, such as genetic studies and imaging analysis of the brain, are treated with similar oral preparations of  $\beta$ -amyloid peptide.

Pharmaceutical formulations for oral or enteral administration to treat Alzheimer's disease are prepared  
25 from a composition comprising a  $\beta$ -amyloid peptide and a pharmaceutically acceptable carrier suitable for oral ingestion. The quantity of  $\beta$ -amyloid peptide may be between 0.01 mg and 1000 mg per day. In one embodiment of the invention, the dose is greater than 0.05 mg per day. In  
30 another embodiment the dose is greater than 0.1 mg per day, and may be greater than 1 mg per day. In a further embodiment, the dose is between 0.05 and 1 mg per day. However, the total dose required for treatment can vary according to the individual and the severity of the  
35 condition. This amount can be further refined by well-known methods such as establishing a matrix of dosages and frequencies of administration.

For by-inhalation administration (i.e. delivery to the bronchopulmonary mucosa) suitable sprays and aerosols

can be used, for example using a nebulizer such as those described in U.S. Patent Nos. 4,624,251 issued November 25, 1986; 3,703,173 issued November 21, 1972; 3,561,444 issued February 9, 1971 and 4,635,627 issued January 13, 1971. The aerosol material is inhaled by the subject to be treated.

Other systems of aerosol delivery, such as the pressurized metered dose inhaler (MDI) and the dry powder inhaler as disclosed in Newman, S.P. in Aerosols and the Lung, Clarke, S.W. and Davia, D. eds. pp. 197-224, Butterworths, London, England, 1984, can be used when practicing the present invention.

Aerosol delivery systems of the type disclosed herein are available from numerous commercial sources including Fisons Corporation (Bedford, MA), Schering Corp. (Kenilworth, NJ) and American Pharmoseal Co. (Valencia, CA).

Formulations for nasal administration can be administered as a dry powder or in an aqueous solution. Preferred aerosol pharmaceutical formulations may comprise for example, a physiologically-acceptable buffered saline solution containing the  $\beta$ -amyloid antigen of the present invention.

Dry aerosol in the form of finely divided solid  $\beta$ -amyloid particles that are not dissolved or suspended in a liquid are useful in the practice of the present invention. The antigen may be in the form of dusting powders and comprise finely divided particles having an average particle size of between about 1 and 5  $\mu$ m, preferably between 2 and 3  $\mu$ m. Finely divided antigen particles may be prepared by pulverization and screen filtration using techniques well known in the art. The particles may be administered by inhaling a predetermined quantity of the finely divided material, which can be in the form of a powder.

Specific non-limiting examples of the carriers and/or diluents that are useful in the pharmaceutical formulations of the present invention include water and physiologically-acceptable buffered saline solutions such as phosphate buffered saline solutions pH 7.0-8.0.

The nasally administered formulation of the present invention may include a thermosetting gel which

increases in viscosity at body temperature upon contact with the nasal mucosa.

Formulations for buccal administration can include mucoadhesive mixed with effective amounts of the  $\beta$ -amyloid antigen. Effective amounts are anticipated to vary according to the formulation employed. For formulation administered by inhalation, the effective amount is likely to be less than that of the oral dose.

The duration of treatment in humans should be a minimum of two weeks, and typically three months, and may be continued indefinitely or as long as benefits persist. The treatment may be discontinued if desired (in the judgment of the attending physician) and the patient monitored for signs of relapse. If clinical symptoms or other disease indicators show that the patient is relapsing, treatment may resume.

As will be understood by those skilled in the art, the dosage will vary with the antigen administered and may vary with the sex, age, and physical condition of the patient as well as with other concurrent treatments being administered. Consequently, adjustment and refinement of one or both of the dosages used and the administration schedules will preferably be determined based on these factors and especially on the patient's response to the treatment. Such determinations, however, require no more than routine experimentation, as illustrated in Examples provided below.

Administration of  $\beta$ -amyloid antigens can be conjoined with mucosal administration of one or more enhancers, i.e. substances that enhance one or more beneficial effect of the  $\beta$ -amyloid antigens. Such enhancers include LPS, Lipid A (as described in U.S. Application Serial No. 08/202,677, published as WO 91/01333) IL-4, IL-10 and Type I interferons, including  $\alpha$ -interferon and  $\beta$ -interferon (See, e.g. U.S. Application Serial No. 08/420,980 and 08/420,979 and WO 95/27499 and WO 95/27500), cholera toxin B-subunit, and LTB (heat labile enterotoxin). Experiments have been carried out, described further below, in which administration of IL-4 in conjunction with  $\beta$ -

amyloid peptide was found to be particularly effective. As used in this paragraph, "conjoined with" means before, substantially simultaneously with, or after administration of these antigens. Naturally, administration of the conjoined substance should not precede nor follow administration of the antigen by so long an interval of time that the relevant effects of the substance administered first have worn off. Therefore, enhancers should usually be administered within about 24 hours before or after the  $\beta$ -amyloid antigens and preferably within about one hour.

The invention is further described below by reference to examples, the purpose of which is to illustrate the present invention without limiting its scope. All documents cited herein are incorporated by reference.

15

### Example 1

#### Experimental Animals

Female BALB/C (6-8 weeks of age); DBA/2; C3H/eb; C47b1/6; SWISSXBDF1 or SHL/J mice were used. The animals were maintained in a temperature- and light-controlled environment with free access to feed and water. Each experimental group contained no fewer than 5 mice.

20

#### Immunization

Mice were immunized with 100  $\mu$ g of [1-40] $\beta$ -amyloid peptide ([1-40]A $\beta$ ) emulsified in 50  $\mu$ g CFA (Difco Laboratories, Inc.). On day 10, popliteal lymph nodes and spleens were removed and for T-cell proliferation and cytokine production.

25

#### Proliferation Assay

Cells ( $5 \times 10^5$  per well) were cultured in triplicate in 96-well round-bottomed plates (Falcon<sup>TM</sup>, Becton Dickinson, Lincoln Park, New Jersey) in the presence of antigen (1-100  $\mu$ g/ml) for 72 hr. [<sup>3</sup>H]Thymidine (1  $\mu$ Ci/well) was added for the last 7 hr of culture before harvesting the cells. Incorporation of [<sup>3</sup>H]thymidine was determined using a

30



Betaplate scintillation counter (Beckman Instruments Inc., Fullerton, California). The proliferation response was measured as  $\Delta\text{cpm}$  ( $\text{cpm}[\text{test}] - \text{cpm}[\text{control without antigen}]$ ). The stimulation index (SI) was calculated as  $\text{mean cpm}[\text{with antigen}] / \text{mean cpm}[\text{without antigen}]$ .

Figure 1A shows significant suppression of proliferation of spleen cells from SWISSXBDF1 mice (nontransgenic littermates of Alzheimer's model) either fed (75  $\mu\text{g}/\text{feeding} \times 5$ ) or nasally treated (25  $\mu\text{g}/\text{direct}$  application to nasal mucosa  $\times 3$ ) with [1-40] $\beta$ -amyloid peptide and immunized two days after the last treatment with 100  $\mu\text{g}$  [1-40] $\beta$ -amyloid peptide in 50  $\mu\text{g}$  CFA per mouse. Figure 1B shows suppression of proliferation in popliteal lymph nodes from SWISSXBDF1 mice either fed or nasally treated with [1-40] $\beta$ -amyloid peptide determined ten days after being immunized in the foot pad with 100  $\mu\text{g}$  [1-40] $\beta$ -amyloid peptide in 50  $\mu\text{g}$  CFA per mouse. The results show that proliferation of spleen and popliteal lymph node T-cells to [1-40]  $\beta$ -amyloid peptide was significantly suppressed in treated mice (fed or nasally administered the same peptide) compared to untreated controls. These results are particularly meaningful given that the cells tested for proliferative response were collected just 10 days after immunization, before the immunization effect subsided.

Figure 2A compares proliferation of popliteal lymph node T-cells from SJL/J, DBA/1, C57BL/6, BALB/C and C3H/EB mice immunized with [1-40] $\beta$ -amyloid peptide. Figure 2B compares proliferation in spleen cells from SJL/J, DBA/1, C57BL/6, BALB/C and C3H/EB mice immunized with [1-40] $\beta$ -amyloid peptide. This experiment shows that many strains of normal mice (not transgenic mice, not Alzheimer's model) respond to [1-40] $\beta$ -amyloid peptide, within a short time after immunization. It should be noted that T-cells for the experiment shown in this Example are stimulated with antigen only once *in vitro* prior to the proliferation assay.

Additional stimulations would enlist response from the apparent non-responder strain C57BL/6 and (DBA/1). This experiment provides information about the responding ability of cells from various strains and was useful in designing

the subsequent experiments described below. It is believed to be advantageous to select "good responders" (such as C3H/EB) but not "too high responders" (SJL/J was thus not chosen).

5

### Example 2

#### Cytokine Production Assays

Levels of IL-4, IL-2, IL-10, TGF- $\beta$  and IFN $\gamma$  in supernatants were determined by capture ELISA as described. Friedman, A., et al., Proc. Nat'l Acad. Sci. U.S.A., **91**, 6688 (1994); Yang, X., et al., J. Immunol., **150**, 4354 (1993). Briefly, supernatants were added to microtiter (Maxisorp) plates, previously coated with rat anti-mouse IL-4, IL-2, IL-10, TGF- $\beta$  or IFN- $\gamma$  monoclonal antibodies (capture antibodies, Pharmingen; 1  $\mu$ g/ml at 4°C over night) and blocked with BSA-diluent/blocking solution (KPL). Plates were washed, and standards and samples were added for another overnight incubation at 4°C. Bound cytokine was detected by the addition of ABTS (Kirkegaard & Perry). Wells were washed, and cytokine levels determined using biotinylated rat anti-mouse IL-4, IL-2, IL-10, TGF- $\beta$  or IFN- $\gamma$  monoclonal antibodies (detecting antibodies, Pharmingen) and a peroxide visualization system (peroxidase-labeled streptavidin). IL-2 and IL-4 were analyzed after 24 hours of cell incubation with antigen, IL-10 and IFN- $\gamma$  after 40 hours and TGF- $\beta$  after 72 hours. Cytokine levels were calculated from a log-log plot of absorbance vs. concentration of recombinant cytokines (Pharmingen), and results are expressed in pg/mL (for IL-4, IL-2, IL-10, TGF- $\beta$  and IFN- $\gamma$ ). Threshold sensitivities of ELISA assays were 5 pg/mL, 10 pg/mL and 2.5 ng/mL for IL-4, IL-2 and IFN- $\gamma$ , respectively.

#### Statistical analysis

The statistical significance of differences between experimental groups was determined using unpaired two-tailed Student's t-test, with differences considered significant at  $P < 0.05$ .

Determination that mice exposed to A $\beta$  peptide either fed or nasally administered display suppression of immunostimulatory (proinflammatory) cytokines (e.g. IL-2 and IFN- $\gamma$ ) and enhancement of immunoregulatory (anti-inflammatory) cytokines (e.g. IL-4, IL-10 and TGF- $\beta$ ) *in vivo*.

C3H/eb mice were fed [1-40]A $\beta$  500  $\mu$ g/feeding, 50  $\mu$ g/feeding, or 5  $\mu$ g/feeding every day for 5 days (200  $\mu$ l/feeding). Nasal administration was given at 50  $\mu$ g/application or 5  $\mu$ g/application every day for 3 days (10  $\mu$ l/application). Two days after the last feeding or nasal administration, fed, nasal or non-fed mice were immunized in the foot pad with 100  $\mu$ g [1-40]A $\beta$  in 50  $\mu$ g CFA per mouse.

Figure 3A provides a comparison of the secretion of cytokines IL-2, IFN- $\gamma$ , IL-4, IL-10 and TGF- $\beta$  in popliteal lymph nodes from SJL/J, DBA/1, C57BL/6, BALB/C and C3H/EB mice immunized with [1-40] $\beta$ -amyloid peptide but not fed or otherwise treated with the peptide. Figure 3B compares the secretion of these cytokines in spleen cells from a similar range of mice strains immunized with [1-40] $\beta$ -amyloid peptide (also not treated with the peptide). Comparison of the results in Fig. 3A and Fig. 3B shows that the T-cells from spleens secrete a different cytokine profile from that of T-cells from popliteal lymph nodes, and that the cytokine profile varies with the strain of mouse. Again, the reason for this experiment is to select responders that will show a difference in the property tested under the experimental conditions.

Figure 4A shows the secretion of the immunoregulatory cytokine IL-10 in popliteal lymph nodes from C3H/EB mice either fed (5, 50 or 500  $\mu$ g/feeding for 5 days) or nasally administered (5 or 50  $\mu$ g/application for 3 days) [1-40] $\beta$ -amyloid peptide prior to immunization (2 days after the last feeding or nasal application) with 100  $\mu$ g [1-40] $\beta$ -amyloid peptide in 50  $\mu$ g CFA. Figure 4B demonstrates the secretion of cytokine IL-10 in spleen cells from C3H/EB mice either fed or nasally administered [1-40] $\beta$ -amyloid peptide under similar conditions. This experiment also

served as a preliminary dose response experiment for this mouse strain. Given that the T-cells underwent a single stimulation *in vitro* and that the effects of the immunization (which would suppress IL-10 levels in all mice tested) had not subsided, the results, which show significant enhancement of IL-10 in at least one of spleen and PLN cells treated with at least one dose, provides a showing that the cytokine profile of T-cells that recognize [1-40] $\beta$  amyloid peptide can be altered by tolerization to the same antigen.

Figure 5A illustrates the result in secretion of the Th1 immunostimulatory (proinflammatory) cytokine IL-2 in popliteal lymph nodes from C3H/EB mice at various feeding levels (5, 50 and 500  $\mu$ g/feeding for 5 days) and when nasally administered (5 or 50  $\mu$ g/application for 3 days) [1-40] $\beta$ -amyloid peptide prior to immunization (2 days after the last feeding or nasal application) with 100  $\mu$ g [1-40] $\beta$ -amyloid peptide in 50  $\mu$ g CFA. Figure 5B similarly shows the secretion of cytokine IL-2 in spleen cells from C3H/EB mice either fed (at lower doses, e.g., 5  $\mu$ g/feeding for 5 days) or nasally administered (at 50  $\mu$ g/application for 3 days) [1-40] $\beta$ -amyloid peptide prior to immunization (2 days after the last feeding or nasal application) with 100  $\mu$ g [1-40] $\beta$ -amyloid peptide in 50  $\mu$ g CFA. The fact that IL-2 secretion is suppressed at least at one dose of treatment in either the spleen or the PLN T-cells shows suppression of Th1 responses despite the nonoptimal (i) timing, of the experiment (relative to the time of immunization); (ii) amount fed; and (iii) stimulation of cells *in vitro* (only once). Suppression of IL-2 secretion was clear by nasal administration (Fig. 5A) and at 5  $\mu$ g fed and 50  $\mu$ g nasal (Fig. 5B). Based on the results for Fig. 5A, and Figs. 5B, 6A, 6B, and 7A, 7B, it was decided to use 25  $\mu$ g nasally and 75  $\mu$ g orally in subsequent experiments. These are not optimal amounts, but they provide a quantity for demonstrating differences within the experimental conditions employed. See Figs. 8A and 8B below.

Figure 6A shows secretion of TGF- $\beta$  in popliteal lymph nodes from C3H/EB mice either fed (at lower doses; 5 or 50  $\mu$ g/feeding for 5 days) or nasally administered (at 50  $\mu$ g/application for 3 days) [1-40] $\beta$ -amyloid peptide prior to immunization (2 days after the last feeding or nasal application) with 100  $\mu$ g [1-40] $\beta$ -amyloid peptide in 50  $\mu$ g CFA. Figure 6B illustrates the enhanced secretion of TGF- $\beta$  in spleen cells from C3H/EB mice either fed (at lower dose; 5 and 50  $\mu$ g/feeding for 5 days) or nasally administered (at 50  $\mu$ g/application for 3 days) [1-40] $\beta$ -amyloid peptide prior to immunization (2 days after the last feeding or nasal application) with 100  $\mu$ g [1-40] $\beta$ -amyloid peptide in 50  $\mu$ g CFA.

### Example 3

#### Cytokine profiles after feeding or nasal administration and immunization of non-transgenic littermates of the transgenic mouse model of Alzheimer's disease.

**Feeding and Immunization:** SWISSXXB6D2F1 mice were fed with [1-40]A $\beta$  75  $\mu$ g/feeding x 5 or nasally administered with 25  $\mu$ g/application x 3, and 2 days after the last treatment, were immunized with 100  $\mu$ g [1-40]A $\beta$  in 50  $\mu$ g CFA. Ten days later, the popliteal lymph node and spleen T-cells were checked for cytokine production as described above. The results of these experiments demonstrated that mice pretreated with A $\beta$  peptide and immunized with A $\beta$  peptide had a reduced *in vitro* response to A $\beta$  stimulation for proinflammatory (Th1) cytokines (and IFN- $\gamma$  and IL-2 at least pursuant to nasal administration of 25  $\mu$ g) and an increased response for anti-inflammatory (Th2) cytokines (TGF- $\beta$  and IL-10). The absence of detected TGF- $\beta$  in non-treated mice in Figure 8B is particularly significant. These observations confirmed studies disclosed in Examples 1-2 on normal mice of several strains showing increased anti-inflammatory cytokine responses as well as decreased PLN proliferation in mice of certain strains (e.g., C3H/EB and DBA/1) which had been mucosally tolerized with A $\beta$  peptide.

Example 4Feeding, nasal administration and immunization of heterozygous PDAPP transgenic mice (animal model of Alzheimer's disease).

Transgenic PDAPP mice were acquired from Athena Neurosciences, Inc. (South San Francisco, CA). These mice express a human gene encoding a mutant form of  $\beta$ -amyloid precursor protein and progressively develop  $\beta$ -amyloid deposits in the hippocampus and cerebral cortex in a manner similar to that seen in Alzheimer's disease. The  $\beta$ -amyloid deposits in the transgenic mice become associated with activated microglial cells, reactive astrocytes and other features of the periplaque inflammatory response seen in Alzheimer's disease brains.

4 to 5 month old PDAPP mice were fed or nasally administered either [1-40] $\beta$ -amyloid peptide or a control protein (myelin basic protein or ovalbumin in amounts of 50 or 500  $\mu$ g per feeding). Administration was carried out three (nasal) or five times consecutively (oral) during an initial one week period, and then on a weekly basis until the animals were between 11 and 12 months old. The orally administered  $\beta$ -amyloid peptide was in a dosage of 10 or 100  $\mu$ g, and the nasally administered  $\beta$ -amyloid was in a dosage of 5 or 25  $\mu$ g.

The feeding and nasal administration of  $\beta$ -amyloid peptide, or ovalbumin to PDAPP mice was conducted among the following mice groups:

Group 0: Non treated; mice nos. 0.1-0.7

Group 1: fed [1-40] $\beta$ -amyloid peptide 100 $\mu$ g/feeding; mice nos. 1.1-1.9

Group 2: fed [1-40] $\beta$ -amyloid peptide 10 $\mu$ g/feeding; mice nos. 2.1-2.9

Group 3: nasal [1-40] $\beta$ -amyloid peptide 25 $\mu$ g/treatment; mice nos. 3.1-3.9

Group 4: nasal [1-40] $\beta$ -amyloid peptide 5 $\mu$ g/treatment; mice nos. 4.1-4.7

Group 5: fed ovalbumin 500 $\mu$ g/feeding; mice nos. 5.1-5.6

Group 6: nasal ovalbumin 50 $\mu$ g/treatment; mice nos. 6.1-6.6  
Group 7: fed MBP 500 $\mu$ g/feeding; mice nos. 7.1-7.5  
Group 8: nasal MBP 50 $\mu$ g/treatment; mice nos. 8.1-8.6

Two mice from each of groups 1, 5, and 8 were  
5 immunized with  $\beta$ -amyloid peptide 10 days prior to sacrifice.

At the end of the treatment period, the mice were  
sacrificed and brains, popliteal lymph nodes and spleens  
removed. Brains were removed and immersed in 70% ethanol  
10 fixative following by paraffin embedding and sectioning.  
One hemisphere was frozen and cryostat-sectioned.

Thereafter, sections of the brains of the orally  
treated mice were examined using antibodies and histological  
stains. Antibodies to glial fibrillary acidic protein  
15 (GFAP) were employed to show abnormal astrocytes, and  
antibody to amyloid precursor protein employed to show  
abnormal neurites. Lymphocytes were examined for cytokine  
production *in vitro* in the presence of  $\beta$ -amyloid.

Results obtained in the examination of brain  
20 sections are shown in Figure 9A and Figure 9B.  
Specifically, results are shown for both visual and  
computer assisted image analysis of  $\beta$ -amyloid plaque load  
for the 8 experimental groups of mice that were tested;  
values are shown for each mouse in the relevant group. The  
25 computer analysis of plaque load represents the percent area  
identified as plaque. The visual assessment of plaque load  
was scored according to the table shown at the bottom of  
Figure 9A.

These results demonstrated a difference in the  
30 development of  $\beta$ -amyloid deposits in the mice treated with  
 $\beta$ -amyloid as opposed to the control groups, with the  
exception of certain ova treated groups, as discussed  
further below, i.e., the treated groups developed fewer  $\beta$ -  
amyloid deposits. The difference was statistically  
35 significant for mice that were nasally administered 25  $\mu$ g of  
 $\beta$ -amyloid versus controls. P values calculated using the 1-  
tailed Mann-Whitney U-test for various group comparisons are  
shown in Figure 10.

Results of immunoreactivity tests conducted on the sections are also shown in Figure 9A and 9B, showing immunoreactivity (IR) of specific antibodies with cells bearing the immunological markers, or secreting the cytokines, shown. Results are shown in particular for the presence of: Mac-1, a marker for activated antigen presenting cells, such as microglia; F4/80, a marker for antigen presenting cells; and MHC-II. Results are also shown for immunoreactivity with the inflammatory cytokines IFN- $\gamma$ , TFN- $\alpha$ , and for the anti-inflammatory cytokines IL-4, IL-10, and TGF- $\beta$ . These data show that the mice treated mucosally with  $\beta$ -amyloid exhibited decreased microglial activation in the brain and decreased production of inflammatory cytokines.

Results of immunohistochemistry experiments conducted on mouse antisera are shown in Figure 11. In these experiments, the antisera were tested in an immunoassay for the presence of IgG subtypes against  $\beta$ -amyloid. In the immunoassay, Down syndrome brain sections, which contain large amounts of  $\beta$ -amyloid, were employed as "capture" antigen. IgG in the mice antisera were bound to the sections, and the presence of IgG subtypes in the bound antibodies distinguished by secondary ( $2^0$ ) antibodies specific for each subtype. These results demonstrated an increase in IgG1 class antibodies against  $\beta$ -amyloid in antisera of mice that were nasally treated with 25  $\mu$ g of  $\beta$ -amyloid over that observed in controls. These antibodies are associated with Th2 type cellular immune responses.

Figures 12-17 are bar graphs showing results of *in vitro* experiments in which PLN from the PDAPP mice that were immunized with [1-40]  $\beta$ -amyloid peptide prior to sacrifice were tested by stimulation with the peptide, and tested for cytokine production. In the animals that were mucosally administered the [1-40]  $\beta$ -amyloid peptide, PLN immune responses were characterized by increased production of IL-10 and TGF- $\beta$  and decreased production of IFN- $\gamma$ , IL-6, and IL-2.



In several of the experiments described above, results were obtained for administration of whole ovalbumin that were substantially different from those obtained using the other controls, and similar to results obtained using  
5 [1-40]  $\beta$ -amyloid. Further experiments were therefore conducted in which it was determined that whole ovalbumin is cross-reactive with [1-40]  $\beta$ -amyloid. Ovalbumin is therefore not an effective control in experiments designed to test immunological properties of [1-40]  $\beta$ -amyloid.

Example 5Administration of  $\beta$ -amyloid In Conjunction with IL-4 or IL-10

Experiments were conducted in which [1-40]  $\beta$ -amyloid was nasally administered to (B6D2)F1 mice in conjunction with nasal administration of IL-4 or IL-10, and prior to immunization of the mice with [1-40]  $\beta$ -amyloid. Specifically, the (B6D2)F1 mice were nasally treated with the following compositions:

- 1) PBS;
- 2) [1-40]  $\beta$ -amyloid;
- 3) IL-4;
- 4) [1-40]  $\beta$ -amyloid+IL-4;
- 5) IL-10; or
- 6) [1-40]  $\beta$ -amyloid+IL-10

The [1-40]  $\beta$ -amyloid was administered in a dose of 25 $\mu$ g and the IL-4 and IL-10 administered in a dose of 1 $\mu$ g. The compositions were administered three times, at intervals of two days. Two days after the last treatment the mice were immunized with 100 $\mu$ g [1-40]  $\beta$ -amyloid in 50 $\mu$ g CFA. Two weeks later splenocytes from the mice were examined for proliferation and cytokine production in the presence of 50 $\mu$ g/ml [1-40]  $\beta$ -amyloid *in vitro*.

Results from these experiments are shown in Figures 18-22. In these experiments, administration of IL-4 was shown to be particularly effective in enhancing suppressive responses induced by  $\beta$ -amyloid peptide administration, as shown, for example in suppression of IFN- $\gamma$  production, increased TGF- $\beta$  production, and increased proliferative responses. The substantial decrease in IL-6 production induced by IL-4 administration in conjunction with  $\beta$ -amyloid peptide is relevant as increased IL-6 production is correlated with Alzheimer's disease.

Example 6Human Treatment

An individual afflicted with Alzheimer's disease is first orally or nasally administered 0.05 mg of [1-40]  $\beta$ -amyloid peptide once a day for a period of one to two weeks at the end of which the patient's cytokine responses are measured using ELISA techniques as described above. If no improvement is observed, the daily dosage is increased (progressively) to 0.1 mg, 0.5 mg, 1 mg, 2.5 mg, 5 mg, 50mg or 100 mg, etc. (and the cytokine responses are monitored weekly or every two weeks) until an effective dosage is determined. (The number of daily doses may also be progressively increased up to six daily either instead of or in addition to an increase in total daily amount of antigen administered.) Once an effective amount and administration schedule has been identified for this patient (over the course of no more than several weeks or longer), therapy continues at this amount and schedule for as long as is beneficial. Periodically, tests of cognitive skill are administered to monitor progress.

Example 7Prevention of Alzheimer's Disease in Humans

An individual is determined to be at risk for contracting Alzheimer's disease by examination of the individual's family history of the disease, and by analysis of magnetic resonance, positron emission tomography, SPECT gamma camera imaging, and/or other imaging of the brain in conjunction with suitable image enhancement agents presently available. In this manner, the presence of amyloid plaque formation in the brain is observed. In addition, the size of the hippocampus is measured and compared with hippocampal size typically seen in development of Alzheimer's disease. These measures provide a reasonable prognosis for increased risk of developing Alzheimer's disease.

An individual at risk for contracting Alzheimer's disease is first orally or nasally administered 0.05 mg of [1-40]  $\beta$ -amyloid peptide once a day for a period of one to

two weeks at the end of which the patient's cytokine and antibody responses are measured using ELISA techniques as described above. If no beneficial effect is observed, the daily dosage is increased (progressively) to 0.1 mg, 0.5 mg, 1 mg, 2.5 mg, 5 mg, 50mg or 100 mg, etc. (and the responses are monitored weekly or every two weeks) until an effective dosage is determined. (The number of daily doses may also be progressively increased up to six daily either instead of or in addition to an increase in total daily amount of antigen administered.) Once an effective amount and administration schedule has been identified for this patient (over the course of no more than several weeks), therapy continues at this amount and schedule for at least 6 months. Periodically, tests of cognitive skill are administered to monitor symptoms of disease.

The invention has been described above by reference to preferred embodiments. It will be understood that various modifications are possible within the scope of the claims that follow. All documents, patents and patent applications referred to in the specification are incorporated by reference in their entirety. In case of conflict, the present disclosure including the present definitions will control.

**What is claimed is:**

1. A method for treating Alzheimer's disease or suppressing the onset of said disease comprising mucosally administering an effective amount of a composition comprising [1-40]  $\beta$ -amyloid peptide or an effective fragment thereof.

2. The method of claim 1 wherein the mammal is a human.

3. A pharmaceutical formulation for administration to a mammal suffering from Alzheimer's disease adapted for mucosal administration and comprising an oral dosage form containing an effective amount of a polypeptide comprising an amino acid sequence of [1-40]  $\beta$ -amyloid peptide, said sequence being greater than 10 amino acids in length, and sufficient to achieve at least one of the following:

- (i) reduce the quantity of one or more proinflammatory Th1 cytokines in the mammal;
- (ii) increase the quantity of one or more anti-inflammatory Th2 or Th3 cytokines in the mammal; or
- (iii) ameliorate, inhibit or delay the onset of at least one clinical symptom of the disease.

4. The pharmaceutical formulation of claim 3 wherein the oral dosage form is a solid dosage form selected from the group consisting of a tablet, a capsule and a caplet.

5. The pharmaceutical formulation of claim 3 wherein the oral dosage form comprises an aqueous suspension solution of  $\beta$ -amyloid peptide.

6. The pharmaceutical formulation of claim 3 further comprising a pharmaceutically acceptable carrier or diluent.

7. A method for treating a mammal suffering from Alzheimer's disease comprising mucosally administering to the mammal an effective amount of a polypeptide comprising an amino acid sequence of  $\beta$ -amyloid peptide and continuing the administration for a period of time sufficient to achieve at least one of the following:

- (i) reduce the quantity of one or more proinflammatory Th1 cytokines in the mammal;
- (ii) increase the quantity of one or more anti-inflammatory Th2 or Th3 cytokines in the mammal; or
- (iii) ameliorate, inhibit or delay the onset of at least one clinical symptom of the disease.

8. A pharmaceutical formulation adapted for mucosal administration to a mammal suffering from Alzheimer's disease comprising an aerosol dosage form containing an effective amount of a polypeptide comprising an amino acid sequence of [1-40]  $\beta$ -amyloid peptide, said sequence being greater than 10 amino acids in length, and sufficient to achieve at least one of the following:

- (i) reduce the quantity of one or more proinflammatory Th1 cytokines in the mammal;
- (ii) increase the quantity of one or more anti-inflammatory Th2 or Th3 cytokines in the mammal; or
- (iii) ameliorate, inhibit or delay the onset of at least one clinical symptom of the disease.

9. The pharmaceutical formulation of claim 8 further comprising a pharmaceutically acceptable carrier or diluent.

10. A method for preventing Alzheimer's disease  
5 in a mammal at risk for contracting the disease comprising orally administering to the mammal an effective amount of a polypeptide comprising an amino acid sequence of  $\beta$ -amyloid peptide and continuing the administration for a period of time sufficient to inhibit or delay onset of at least one of  
10 the following:

- (i) generation of one or more proinflammatory Th1 cytokines in the mammal;
- (ii) decline in the quantity of one or more  
15 anti-inflammatory Th2 or Th3 cytokines in the mammal; or
- (iii) appearance of at least one clinical symptom of the disease.

11. A method for preventing Alzheimer's disease  
20 in a mammal at risk for contracting the disease comprising administering by inhalation to the mammal an effective amount of a polypeptide comprising an amino acid sequence of  $\beta$ -amyloid peptide and continuing the administration for a period of time sufficient to inhibit or delay onset of at  
25 least one of the following:

- (i) generation of one or more proinflammatory Th1 cytokines in the mammal;
- (ii) decline in the quantity of one or more  
30 anti-inflammatory Th2 or Th3 cytokines in the mammal; or
- (iii) appearance of at least one clinical symptom of the disease.

12. A pharmaceutical formulation adapted for  
35 mucosal administration to a mammal at risk for contracting Alzheimer's disease comprising an effective amount of a

polypeptide comprising an amino acid sequence of  $\beta$ -amyloid peptide in an aerosol form sufficient to inhibit or delay onset of at least one of the following:

- 5                   (i)           generation of one or more proinflammatory Th1 cytokines in the mammal;
- (ii)           decline in the quantity of one or more anti-inflammatory Th2 or Th3 cytokines in the mammal; or
- 10                  (iii)          appearance of at least one clinical symptom of the disease.

13. The pharmaceutical formulation of claim 11 further comprising a pharmaceutically acceptable carrier or diluent.

15                   14. A method of treating an individual suffering from, or at risk for suffering from, Alzheimer's disease comprising administering an effective amount of a composition comprising a peptide comprising the amino acid sequence of a fragment of  $\beta$ -amyloid peptide or an analog  
20 thereof effective to suppress immune response associated with said Alzheimer's disease.

15. The method of claim 14 comprising oral administration.

25                   16. The method of claim 15 comprising administration by inhalation.

17. The method of claim 14 comprising administration of  $\beta$ -amyloid peptide.

30                   18. The method of claim 14 wherein said peptide fragment comprises a homologous, conservative, non-peptidic or isosteric replacement for one or more amino acids of  $\beta$ -amyloid peptide.



19. A method of treating Alzheimer's disease comprising administering an effective amount of an analog or mimetic of  $\beta$ -amyloid peptide or of an effective fragment thereof to an individual suffering from said disease.

5           20. A method for treating Alzheimer's disease or suppressing the onset of said disease comprising mucosally administering an effective amount of a peptide composition comprising an amino sequence of  $\beta$ -amyloid protein effective  
10 to suppress said disease, wherein said peptide composition is in a daily dose greater than 0.05 mg.

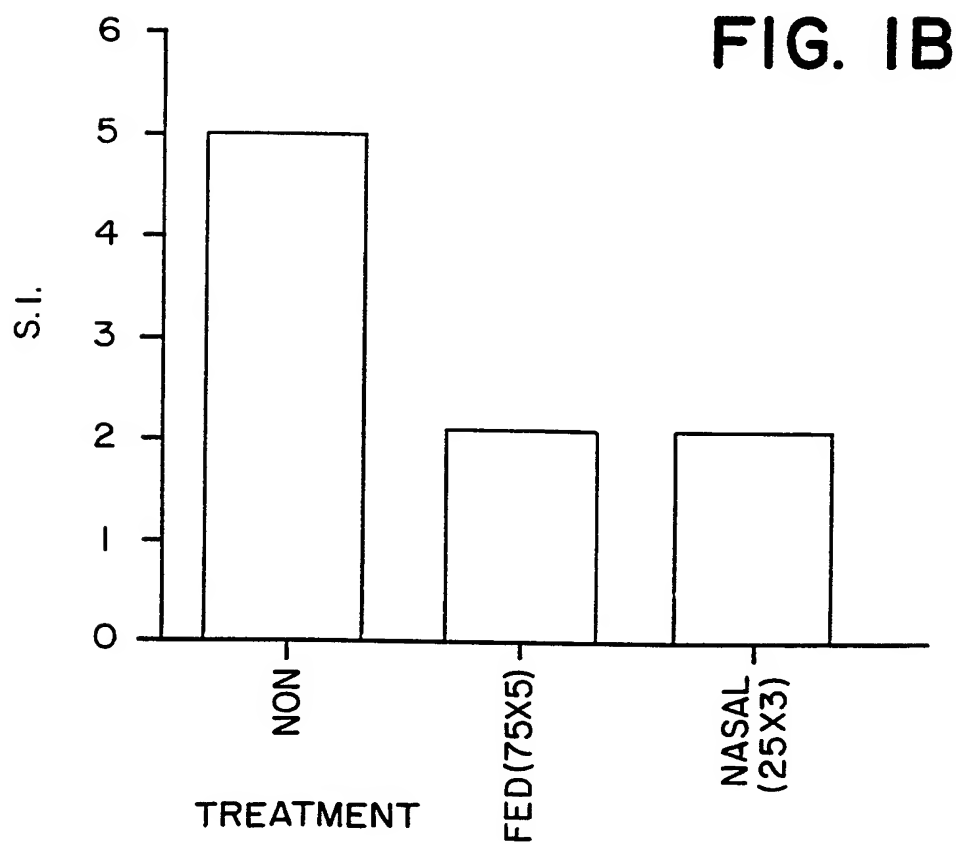
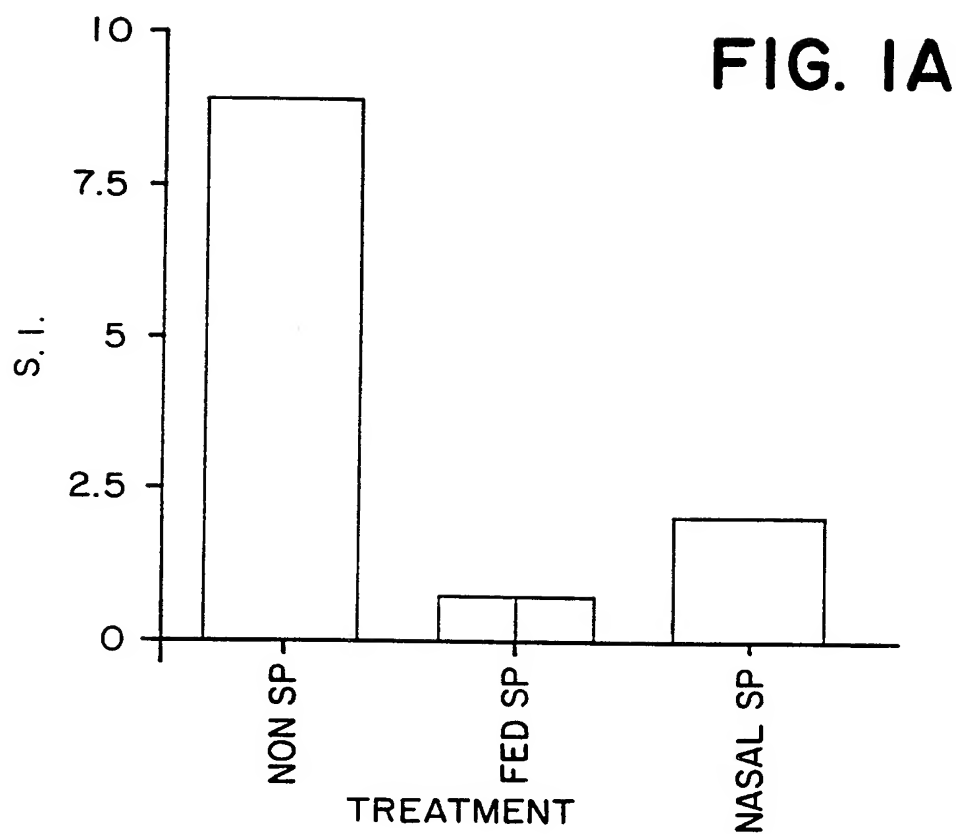
21. The method of claim 20 wherein said administration induces production of anti- $\beta$ -amyloid antibodies.

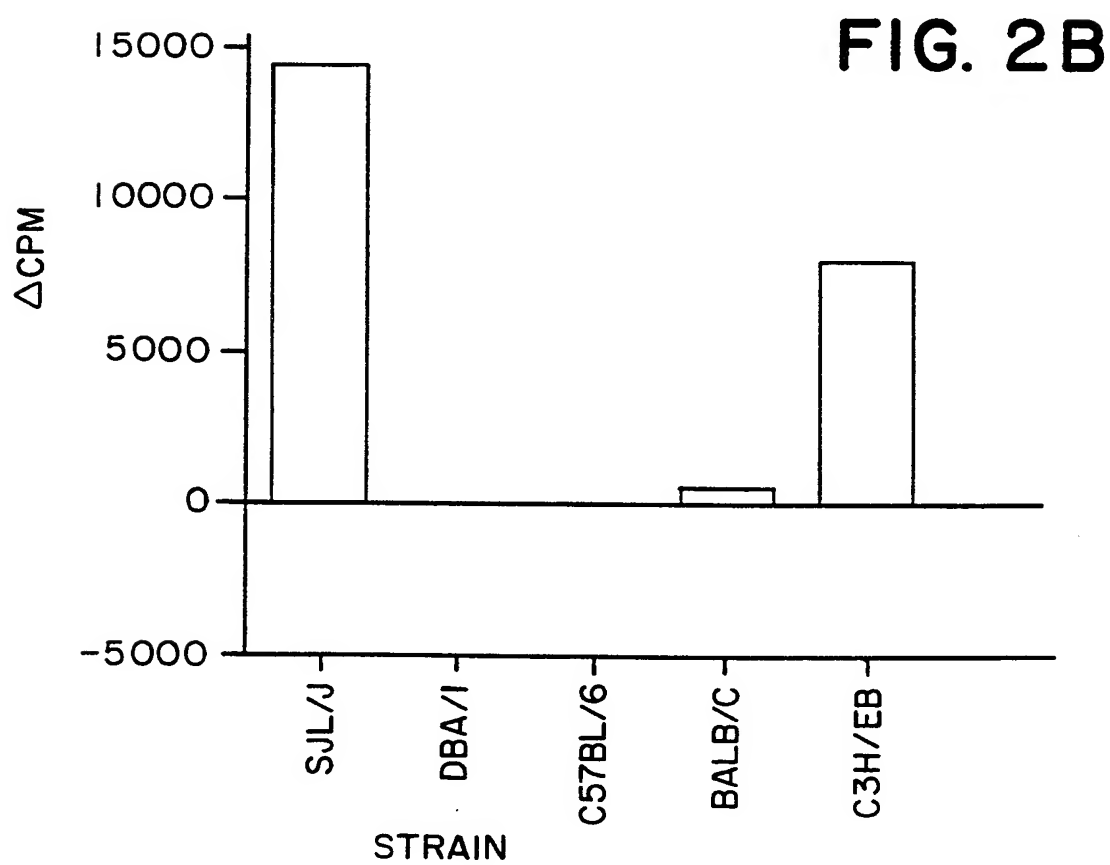
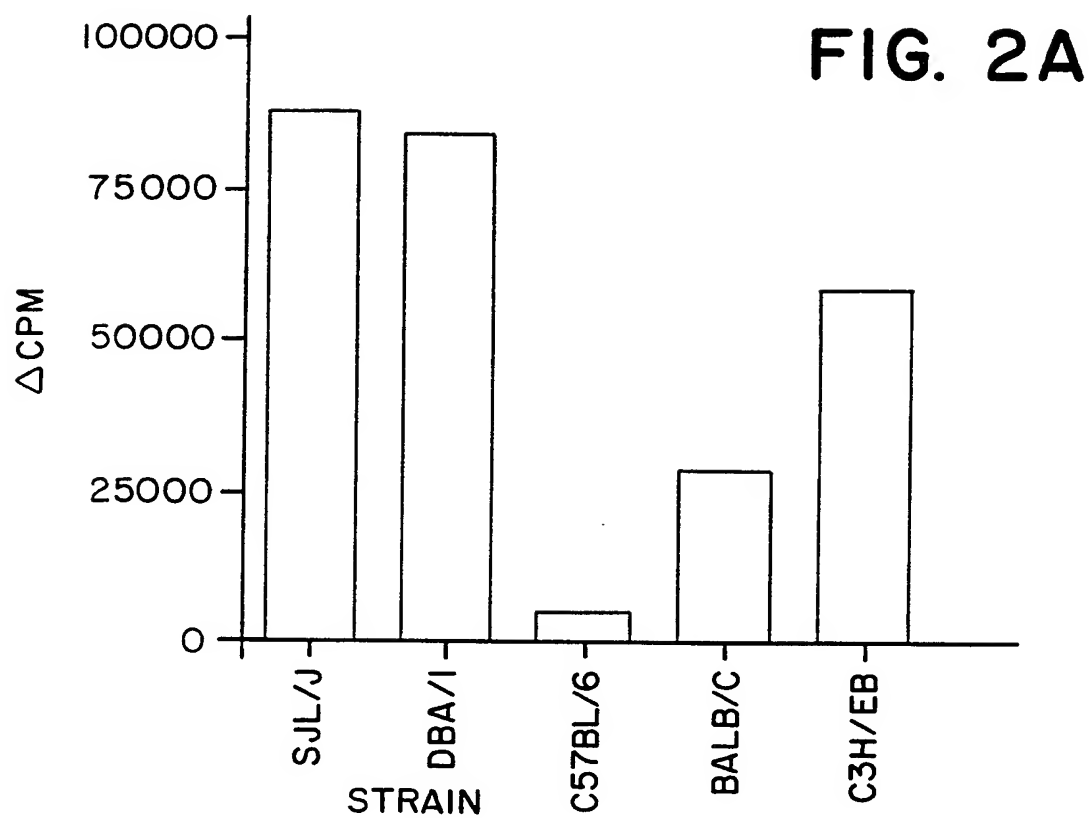
15           22. The method of claim 21 wherein said antibodies are of the IgG1 type.

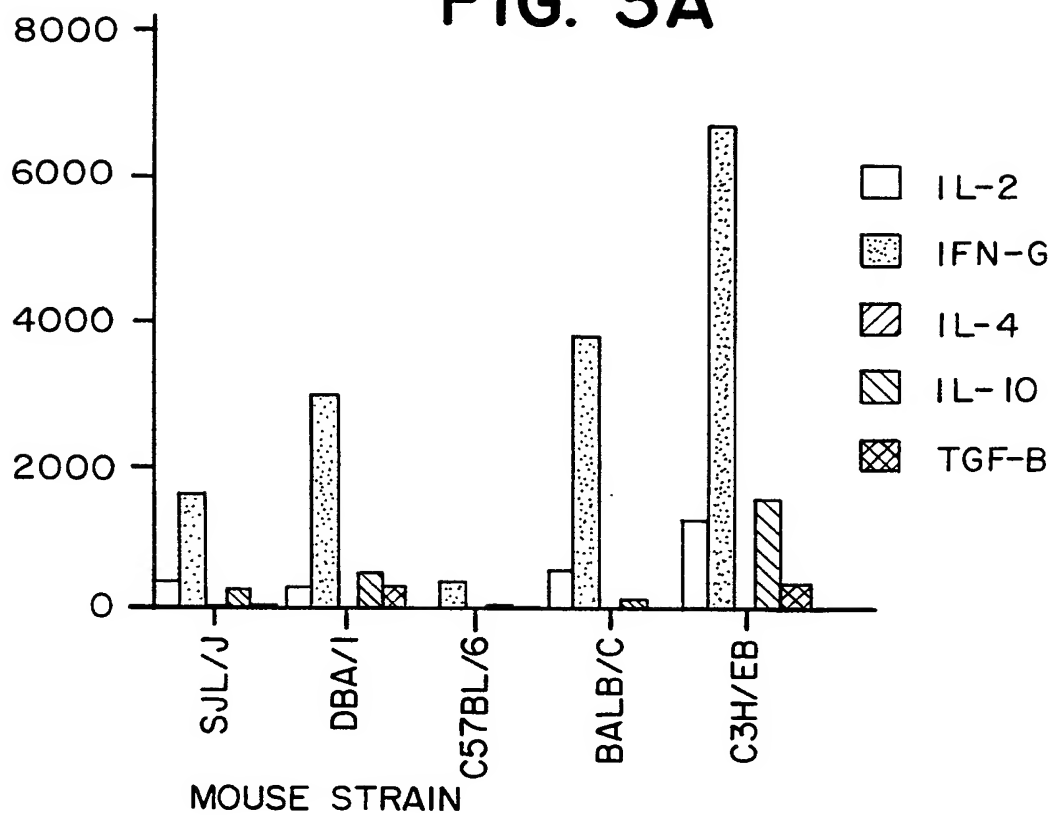
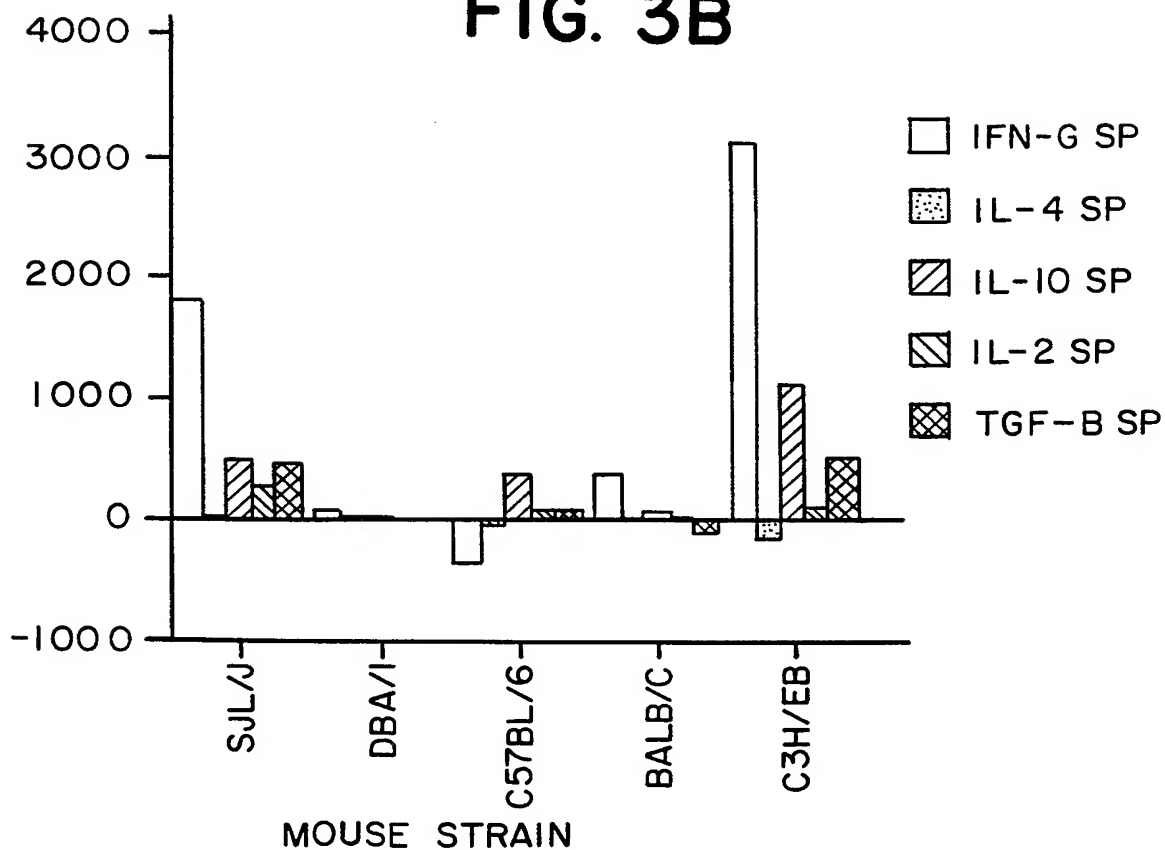
23. The method of claim 20 wherein said administration comprises oral administration that delivers said peptide composition primarily to the gastric mucosa.

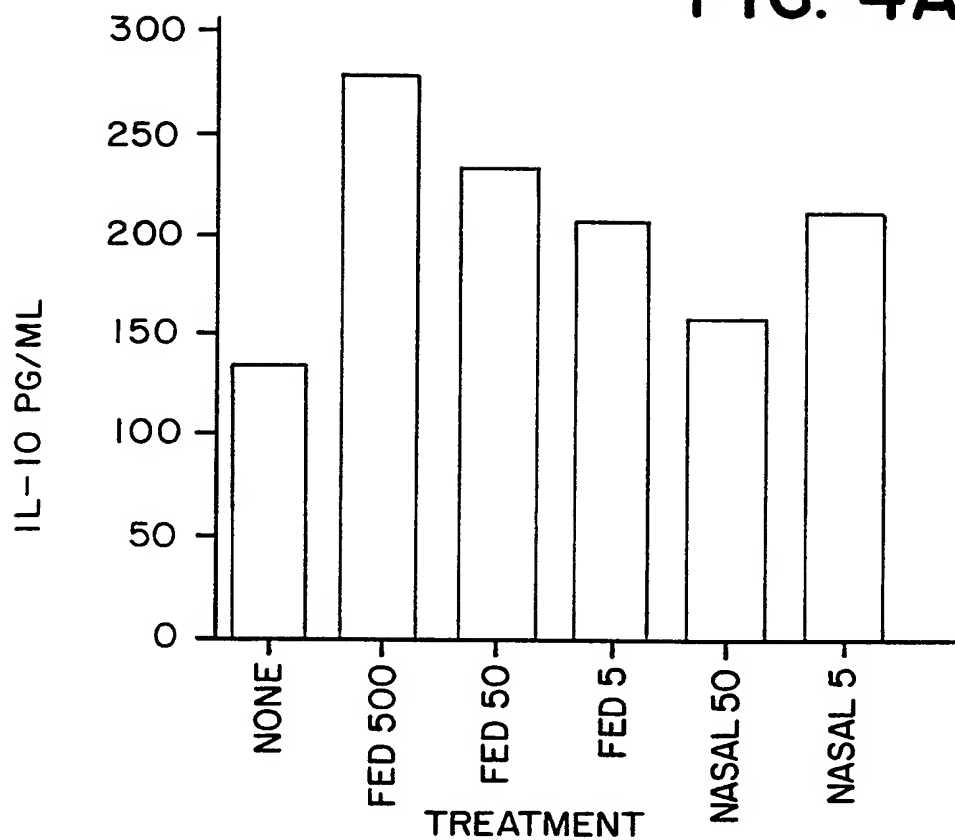
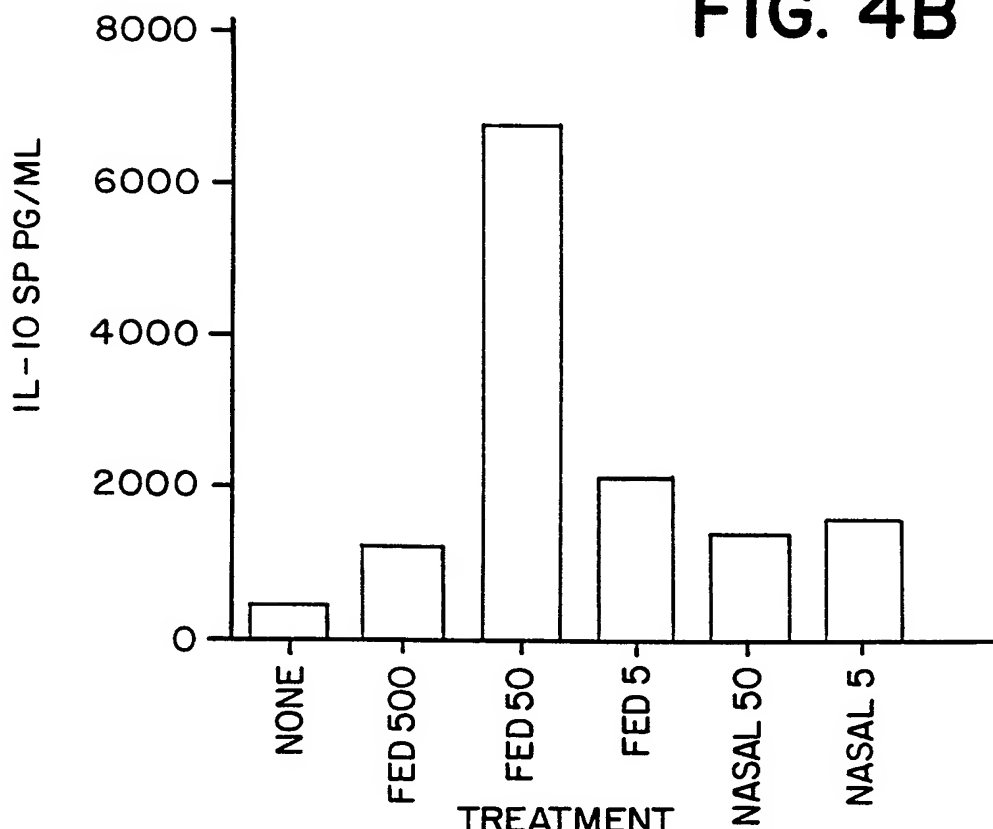
20           24. The method of claim 23 wherein less than 10% of said peptide composition is released in the mouth from a dosage form employed for said administration.

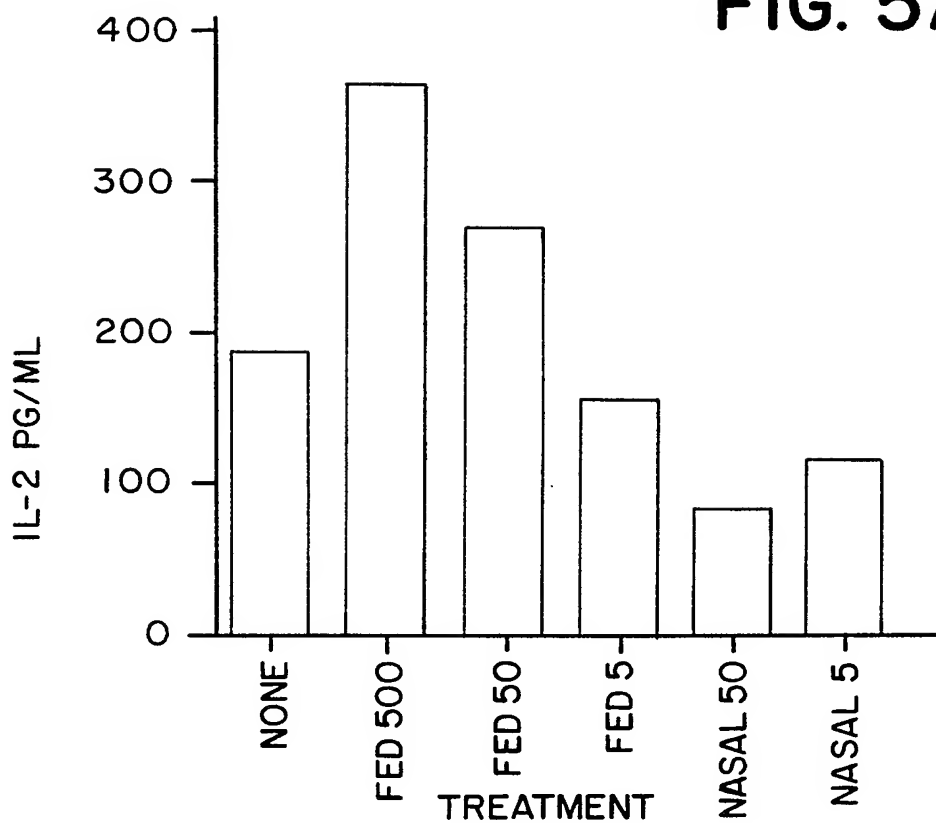
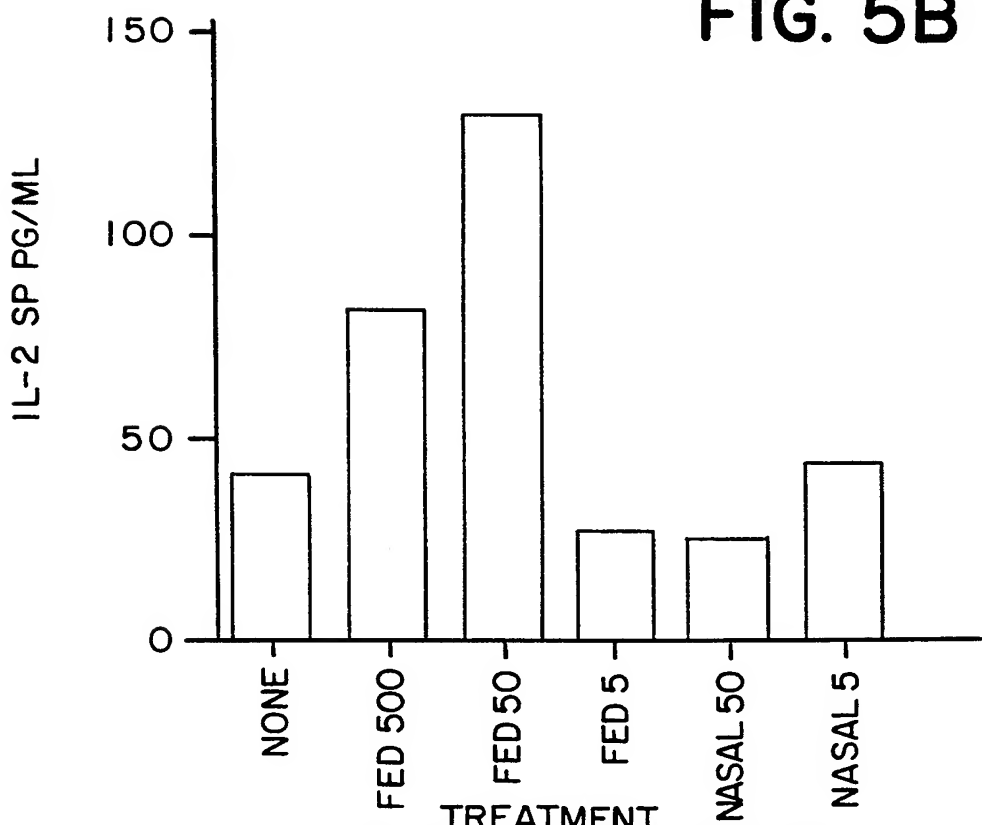
25. The method of claim 20 wherein said administration comprises nasal administration.

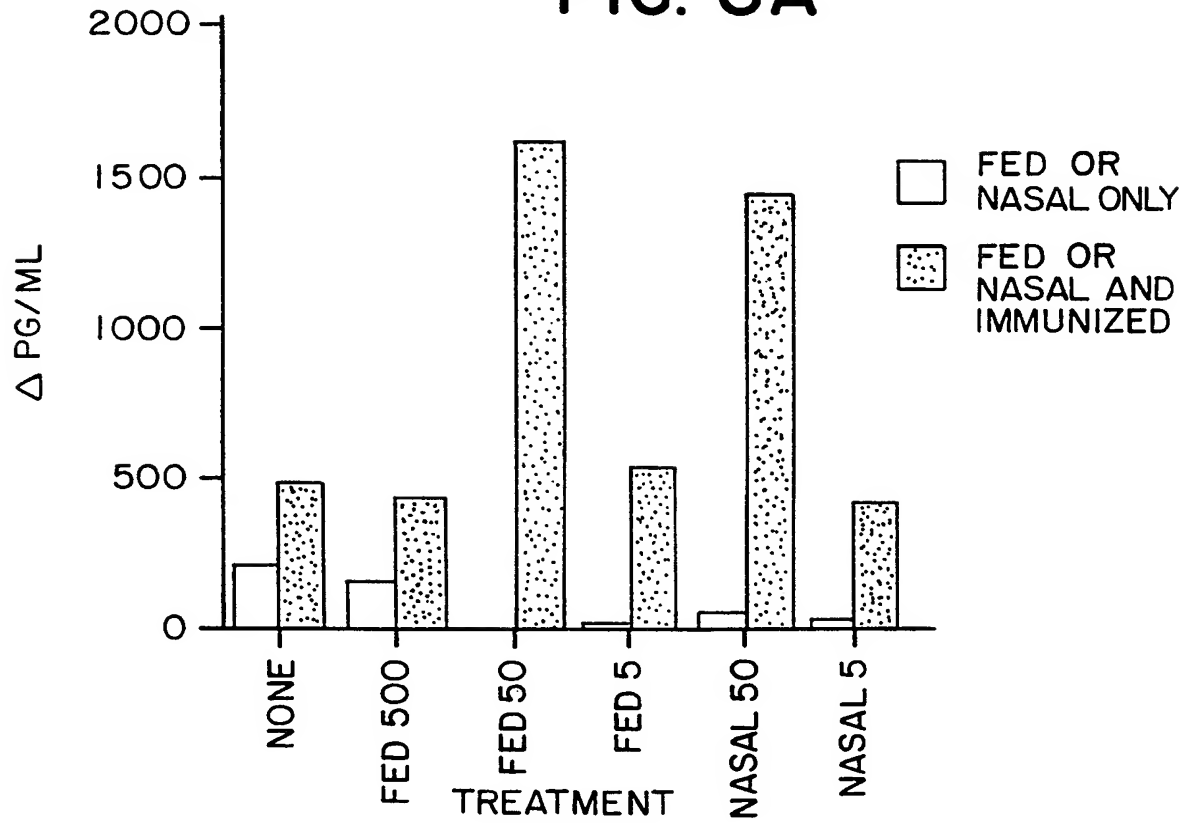
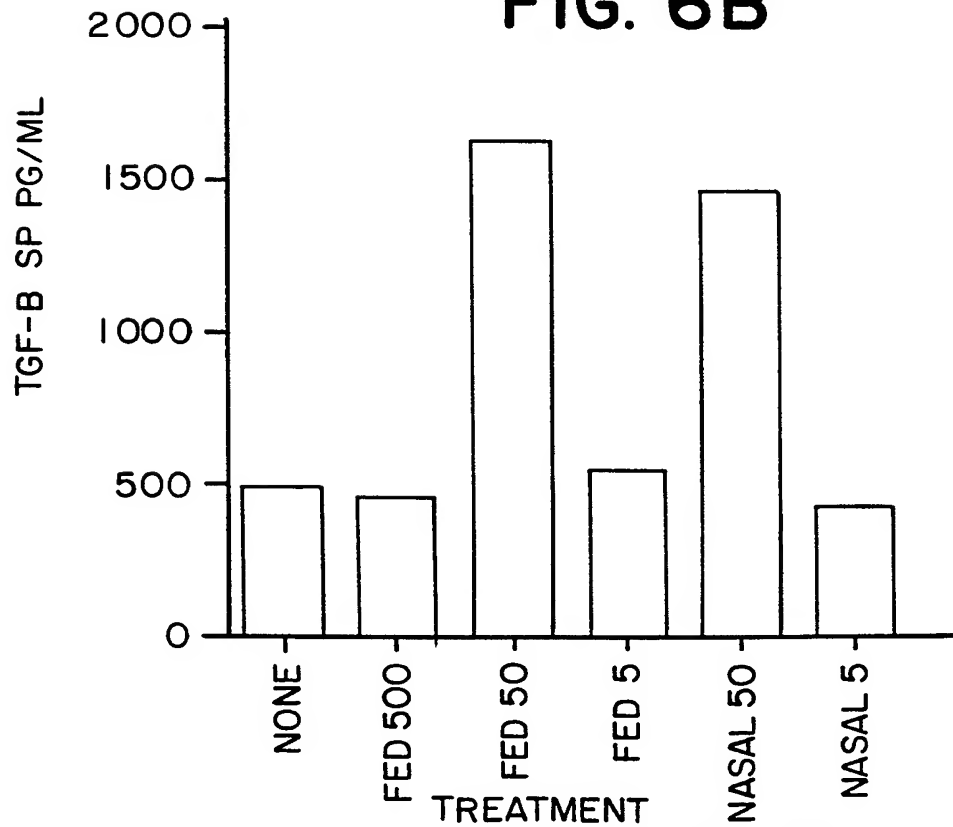


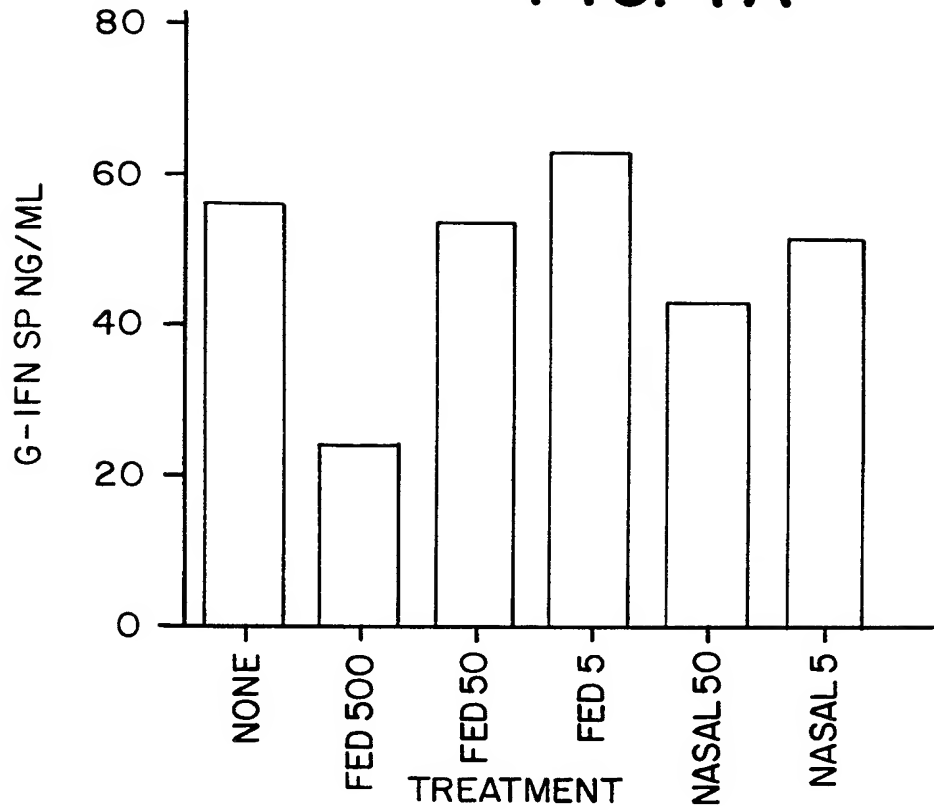
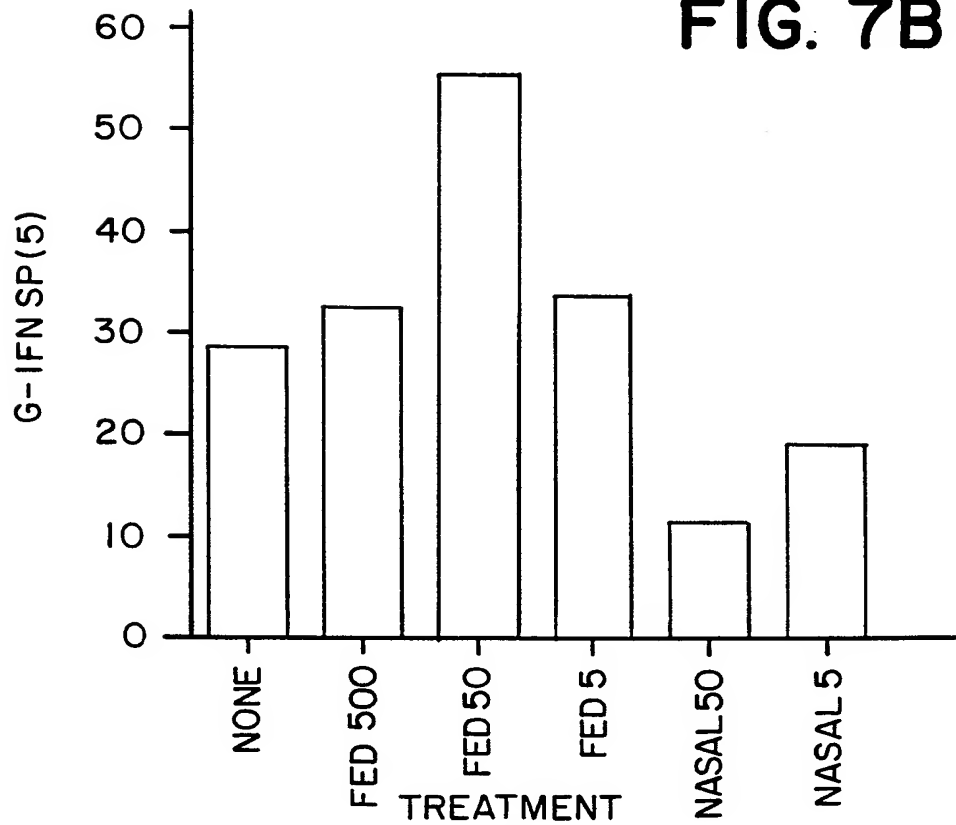


**FIG. 3A****FIG. 3B**

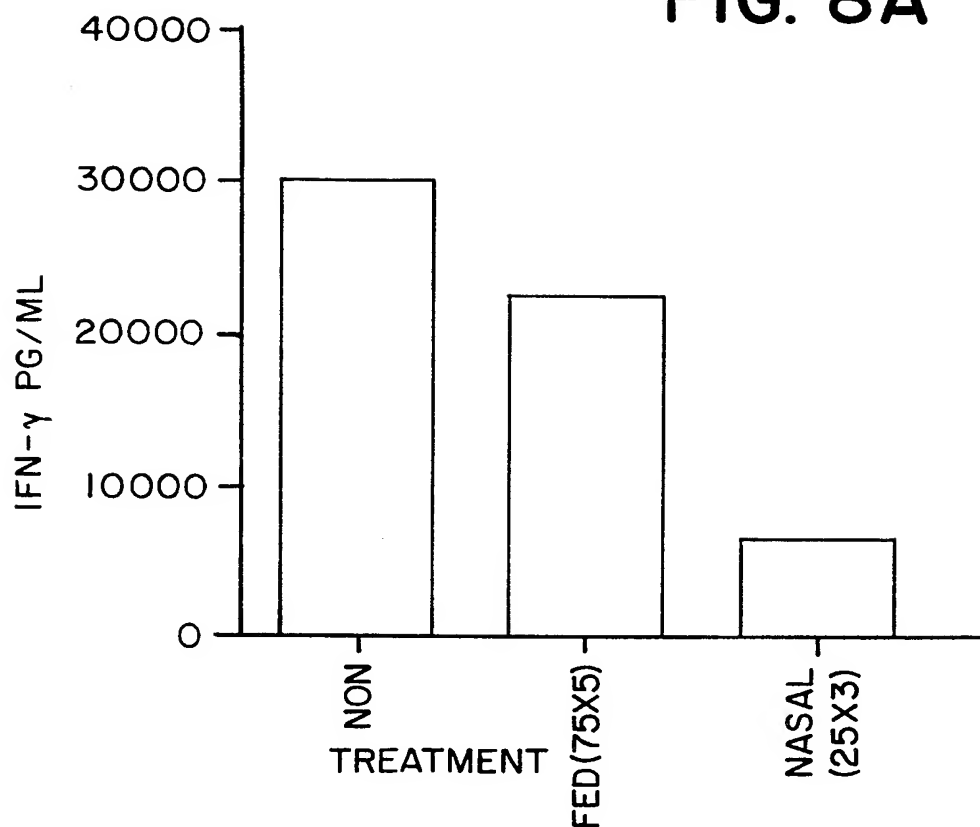
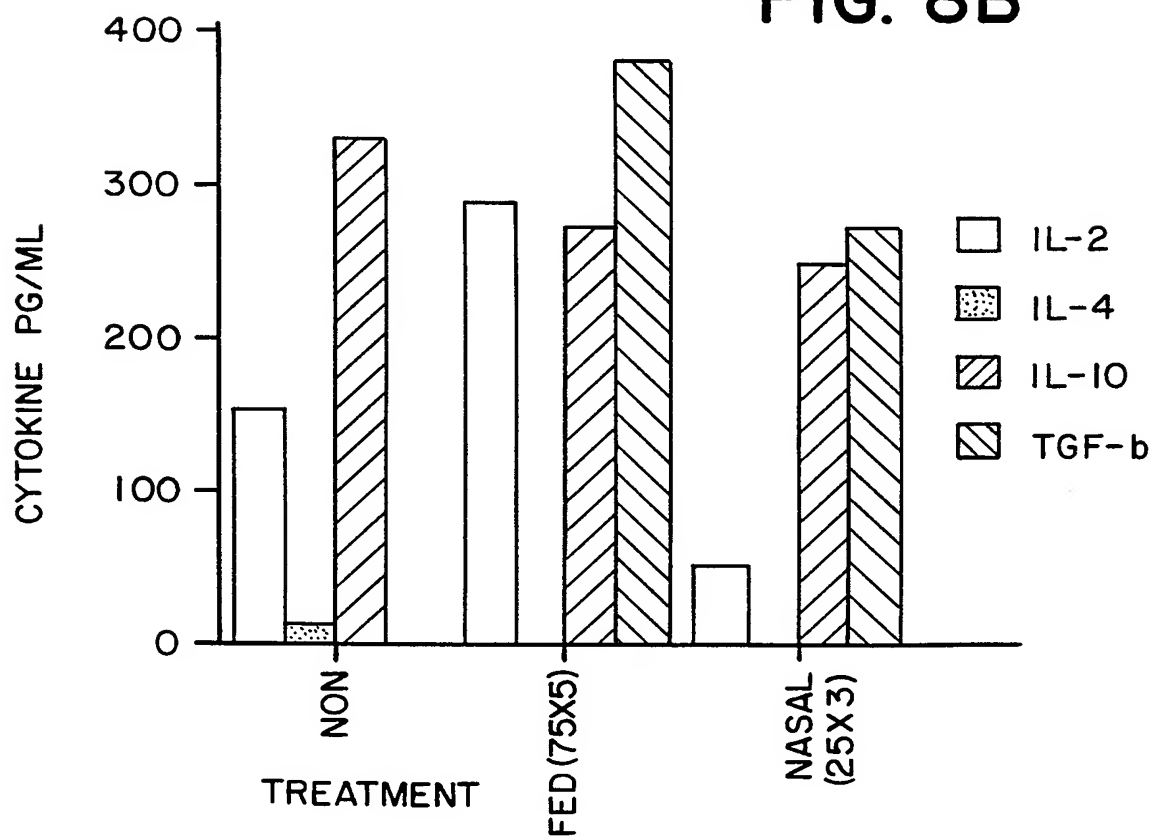
**FIG. 4A****FIG. 4B**

**FIG. 5A****FIG. 5B**

**FIG. 6A****FIG. 6B**

**FIG. 7A****FIG. 7B**



**FIG. 8A****FIG. 8B**

64 PDAPP Mice

Fig. 9A

PAGE 1

| Experimental Group  |     | AB Load: % Area | AB Load               | b     |       |        |               |               |      |       |              |
|---------------------|-----|-----------------|-----------------------|-------|-------|--------|---------------|---------------|------|-------|--------------|
|                     |     | (computer)      | (Visual) <sup>a</sup> | Mac-1 | F4/80 | MHC II | IFN- $\gamma$ | TNF- $\alpha$ | IL-4 | IL-10 | TGF- $\beta$ |
| AB 100 $\mu$ g oral | 1.1 | 0.0466          | 0                     | -     | -     | -      | -             | -             | +    | +     | -            |
|                     | 1.2 | 0.50            | 4                     |       |       |        |               |               |      |       |              |
|                     | 1.3 | 1.0             | 4                     | -     | -     | -      | -             | -             | +    | -     | -            |
|                     | 1.4 | 0.44            | 3                     |       |       |        |               |               |      |       |              |
|                     | 1.5 | 0.12            | 1.5                   | +     | +     | +      | -             | -             | +    | -     | -            |
|                     | 1.6 | 3.24            | 4.5                   |       |       |        |               |               |      |       |              |
|                     | 1.7 | 3.59            | 5                     | -     | -     | -      | +             | +             | +    | -     | -            |
|                     | 1.8 | 2.38            | 5                     |       |       |        |               |               |      |       |              |
|                     | 1.9 | 0.18            | 1                     | -     | +     | -      | -             | -             | +    | ++    | -            |

|                    |     |        |     |   |   |   |   |   |   |   |   |
|--------------------|-----|--------|-----|---|---|---|---|---|---|---|---|
| AB 10 $\mu$ g oral | 2.1 | 0      | 0   | - | - | - | - | - | - | + | - |
|                    | 2.2 | 1.04   | 4   |   |   |   |   |   |   |   |   |
|                    | 2.3 | 0.0267 | 0.5 | - | - | - | - | - | + | - | - |
|                    | 2.4 | 0.32   | 3   |   |   |   |   |   |   |   |   |
|                    | 2.5 | 1.76   | 5   | - | - | - | - | - | - | - | - |
|                    | 2.6 | 3.87   | 5   |   |   |   |   |   |   |   |   |
|                    | 2.7 | 0.90   | 4   | - | - | - | - | - | + | - | - |
|                    | 2.8 | 0.46   | 3   |   |   |   |   |   |   |   |   |
|                    | 2.9 | 0.85   | 3.5 | - | - | - | - | - | - | - | - |

|                     |     |        |     |   |   |   |   |   |   |   |   |
|---------------------|-----|--------|-----|---|---|---|---|---|---|---|---|
| AB 25 $\mu$ g nasal | 3.1 | 0.23   | 2   | - | - | - | - | - | + | + | - |
|                     | 3.2 | 0.0921 | 0.5 |   |   |   |   |   |   |   |   |
|                     | 3.3 | 1.36   | 4.5 | - | - | - | - | - | + | - | - |
|                     | 3.4 | 0.48   | 3   |   |   |   |   |   |   |   |   |
|                     | 3.5 | 0.30   | 3   | - | - | - | - | - | - | - | - |
|                     | 3.6 | 0.71   | 3   |   |   |   |   |   |   |   |   |
|                     | 3.7 | 0.34   | 1   | - | - | - | - | - | + | - | - |
|                     | 3.8 | 0.59   | 2.5 |   |   |   |   |   |   |   |   |
|                     | 3.9 | 0.41   | 1   | - | - | - | - | - | + | - | - |

|                    |     |         |     |   |   |   |   |   |   |   |   |
|--------------------|-----|---------|-----|---|---|---|---|---|---|---|---|
| AB 5 $\mu$ g nasal | 4.1 | 0.38    | 2.5 | - | - | - | - | - | + | - | - |
|                    | 4.2 | 1.93    | 5   |   |   |   |   |   |   |   |   |
|                    | 4.3 | 0.00236 | 0   | - | - | - | - | - | - | - | - |
|                    | 4.4 | 0.0461  | 0.5 |   |   |   |   |   |   |   |   |
|                    | 4.5 | 1.61    | 3.5 | - | - | - | - | - | + | - | - |
|                    | 4.6 | 0.37    | 2.5 |   |   |   |   |   |   |   |   |
|                    | 4.7 | 1.72    | 5   | - | - | - | - | - | + | - | - |

| a   | Scoring                    |
|-----|----------------------------|
| 0   | = none                     |
| 0.5 | = 1 to 4 isolated plaques  |
| 1   | = 5 to 10 isolated plaques |
| 2   | = mild                     |
| 3   | = moderate                 |
| 4   | = moderately severe        |
| 5   | = severe                   |

| b   |                          |
|-----|--------------------------|
| -   | no immunoreactivity (IR) |
| +   | 1-10 IR cells            |
| ++  | 10-100 IR cells          |
| +++ | > 100 IR cells           |

10/18

64 PDAPP Mice

Fig. 9B

PAGE 2

PAGE 2

| Experimental Group |     | AB Load: % Area | AB Load               | b     |       |        |       |       |      |       |       |
|--------------------|-----|-----------------|-----------------------|-------|-------|--------|-------|-------|------|-------|-------|
|                    |     | (computer)      | (Visual) <sup>a</sup> | Mac-1 | F4/80 | MHC II | IFN-γ | TNF-α | IL-4 | IL-10 | TGF-β |
| non-treated        | 0.1 | 0.64            | 3.5                   | +     | +     | +      | +     | +     | ++   | ++    | -     |
|                    | 0.2 | 1.33            | 3.5                   |       |       |        |       |       |      |       |       |
|                    | 0.3 | 0.0761          | 1                     | +     | -     | -      | +     | +     | +    | +     | -     |
|                    | 0.4 | 1.31            | 4.5                   |       |       |        |       |       |      |       |       |
|                    | 0.5 | 0.29            | 2                     | -     | -     | -      | ++    | ++    | ++   | -     | -     |
|                    | 0.6 | 3.59            | 4.5                   |       |       |        |       |       |      |       |       |
|                    | 0.7 | 0.72            | 3                     | +     | +     | +      | ++    | ++    | -    | -     | -     |
| OVA 500 μg oral    | 5.1 | 0.42            | 3                     | -     | -     | -      | -     | -     | +    | +     | -     |
|                    | 5.2 | 0.71            | 3.5                   |       |       |        |       |       |      |       |       |
|                    | 5.3 | 0.89            | 4                     | -     | -     | -      | -     | -     | +    | -     | -     |
|                    | 5.4 | 0.48            | 3                     |       |       |        |       |       |      |       |       |
|                    | 5.5 | 2.96            | 4                     | ++    | ++    | ++     | +     | ++    | +    | ++    | -     |
|                    | 5.6 | 1.28            | 3.5                   |       |       |        |       |       |      |       |       |
| OVA 50 μg nasal    | 6.1 | 0.17            | 2                     | ++    | ++    | +      | -     | -     | +    | +     | -     |
|                    | 6.2 | 0.0682          | 0.5                   |       |       |        |       |       |      |       |       |
|                    | 6.3 | 0.65            | 4                     | -     | -     | -      | -     | -     | +    | -     | -     |
|                    | 6.4 | 0.17            | 1.5                   |       |       |        |       |       |      |       |       |
|                    | 6.5 | 1.41            | 3.5                   | +     | +     | -      | +     | +     | +    | +     | -     |
|                    | 6.6 | 1.97            | 0.5                   |       |       |        |       |       |      |       |       |
| MBP 500 μg oral    | 7.1 | 0.90            | 4.5                   | ++    | ++    | ++     | -     | -     | ++   | +     | -     |
|                    | 7.2 | 0.87            | 4.5                   |       |       |        |       |       |      |       |       |
|                    | 7.3 | 1.09            | 4                     | +     | +     | -      | +     | +     | +    | -     | -     |
|                    | 7.4 | 2.44            | 5                     |       |       |        |       |       |      |       |       |
|                    | 7.5 | 3.76            | 5                     | ++    | +     | +      | +     | +     | +    | -     | -     |
| MBP 50 μg nasal    | 8.1 | 0.24            | 2                     | ++    | ++    | ++     | +     | +     | +    | +     | -     |
|                    | 8.2 | 1.03            | 4                     |       |       |        |       |       |      |       |       |
|                    | 8.3 | 0.32            | 3                     | +     | +     | ++     | +     | +     | -    | -     | -     |
|                    | 8.4 | 1.20            | 4.5                   |       |       |        |       |       |      |       |       |
|                    | 8.5 | 4.07            | 5                     | ++    | ++    | +      | +     | +     | -    | ++    | -     |
|                    | 8.6 | 0.27            | 1                     |       |       |        |       |       |      |       |       |

| a   | Scoring                    |
|-----|----------------------------|
| 0   | = none                     |
| 0.5 | = 1 to 4 isolated plaques  |
| 1   | = 5 to 10 isolated plaques |
| 2   | = mild                     |
| 3   | = moderate                 |
| 4   | = moderately severe        |
| 5   | = severe                   |

| b   |                          |
|-----|--------------------------|
| -   | no immunoreactivity (IR) |
| +   | 1-10 IR cells            |
| ++  | 10-100 IR cells          |
| +++ | > 100 IR cells           |

**AB burden  
Image analysis – HC and Cortex  
1-tailed Mann-Whitney U-test**

Fig. 10

**I. Statistically Significant Results**

P value

|     |   |    |      |               |        |
|-----|---|----|------|---------------|--------|
| Grp | 3 | vs | Grps | 0, 5, 6, 7, 8 | 0.0462 |
| Grp | 3 | vs | Grps | 0, 7, 8       | 0.0380 |
| Grp | 3 | vs | Grps | 7, 8          | 0.0337 |

**II. Not Quite statistically Significant Results**

|      |            |    |      |               |        |
|------|------------|----|------|---------------|--------|
| Grps | 1, 2, 3, 4 | vs | Grps | 0, 5, 6, 7, 8 | 0.0937 |
| Grps | 1, 2, 3, 4 | vs | Grps | 0, 7, 8       | 0.0719 |
| Grps | 1, 2, 3, 4 | vs | Grps | 7, 8          | 0.0645 |
| Grp  | 6          | vs | Grps | 7, 8          | 0.0901 |
| Grps | 3, 4       | vs | Grps | 0, 7, 8       | 0.0583 |
| Grps | 3, 4       | vs | Grps | 0, 5, 6, 7, 8 | 0.0598 |
| Grps | 3, 4       | vs | Grps | 7, 8          | 0.0544 |

**III. Not statistically Significant**

|      |      |    |      |               |        |
|------|------|----|------|---------------|--------|
| Grp  | 6    | vs | Grps | 0, 7, 8       | 0.1254 |
| Grps | 5, 6 | vs | Grps | 7, 8          | 0.1550 |
| Grps | 5, 6 | vs | Grps | 0, 5, 6, 7, 8 | 0.2840 |
| Grps | 3, 4 | vs | Grps | 0, 6, 8       | 0.2811 |
| Grp  | 3    | vs | Grps | 0, 6, 8       | 0.2303 |
| Grp  | 6    | vs | Grps | 1, 2, 3, 4    | 0.4028 |
| Grp  | 8    | vs | Grp  | 0             | 0.1496 |
| Grp  | 3    | vs | Grp  | 1, 2          | 0.1981 |

12/18

## Fig. 11

**Oral Tolerance: Immunohistochemistry of Down's Syndrome Brain Sections using PDAPP mouse antisera**

Each section was pre-treated with formic acid and IgG isotype specific antibodies were used to detect IgG<sub>1</sub>, IgG<sub>2a</sub> and IgG<sub>2b</sub> produced against A $\beta$ .

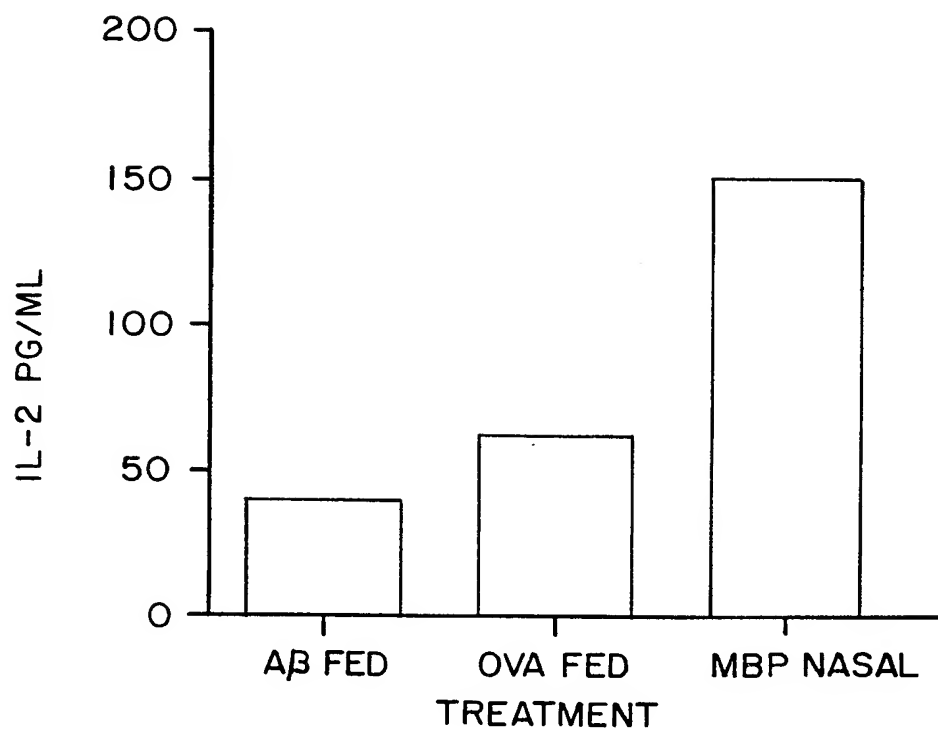
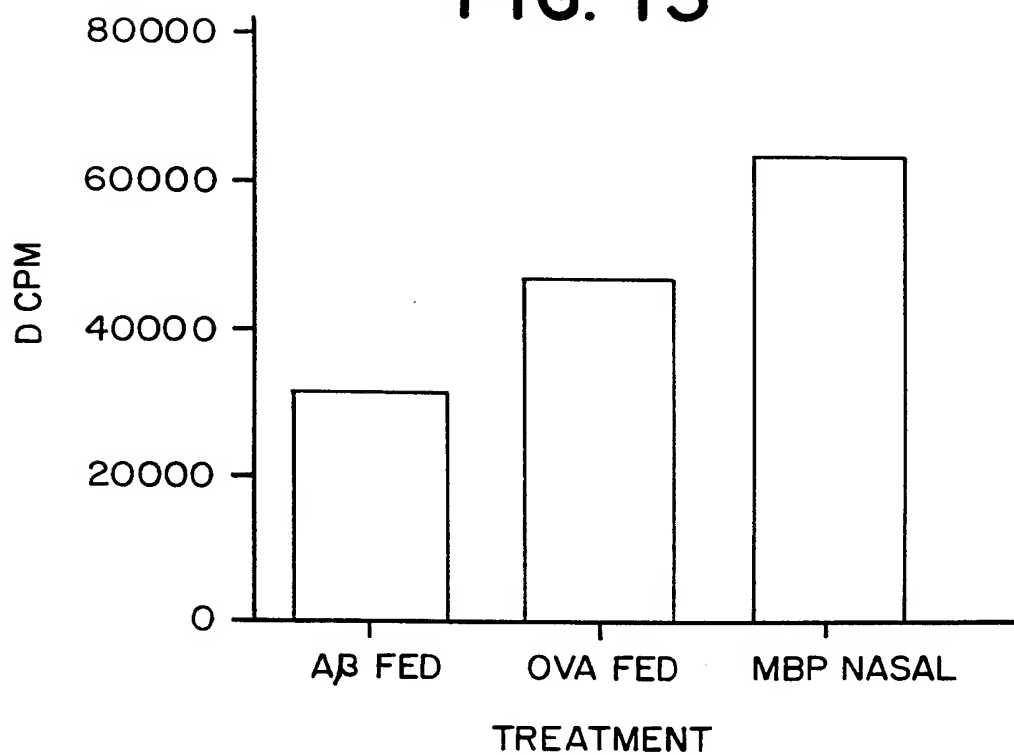
| Mouse antisera<br>(1:10 dilution) | General IgG 2 <sup>0</sup> Ab | IgG <sub>1</sub> 2 <sup>0</sup> Ab | IgG <sub>2a</sub> 2 <sup>0</sup> Ab | IgG <sub>2b</sub> 2 <sup>0</sup> Ab |   |   |
|-----------------------------------|-------------------------------|------------------------------------|-------------------------------------|-------------------------------------|---|---|
|                                   | Plaque staining               | Plaque staining                    | Plaque staining                     | Plaque staining                     |   |   |
| Aβ oral 100mg                     | 1.9*                          | +                                  | +•                                  | -                                   | + |   |
| Aβ oral 10mg                      | 2.7                           | +                                  | -                                   | -                                   | - |   |
|                                   | 2.9                           | ++                                 | +                                   | +                                   | + |   |
| Aβ nasal 25mg                     | 3.1                           | ++                                 | +                                   | -                                   | + |   |
|                                   | 3.2                           | ++                                 | ++•                                 | -                                   | - |   |
|                                   | 3.3                           | ++                                 | +                                   | -                                   | + |   |
|                                   | 3.4                           | ++                                 | +                                   | -                                   | + |   |
|                                   | 3.5                           | -                                  | -                                   | -                                   | - |   |
|                                   | 3.6                           | +                                  | +                                   | -                                   | + |   |
|                                   | 3.7                           | +                                  | +                                   | -                                   | + |   |
|                                   | 3.8                           | -                                  | +                                   | -                                   | - |   |
|                                   | 3.9                           | +                                  | +                                   | -                                   | + |   |
| Aβ nasal 5mg                      | 4.1                           | +                                  | -                                   | -                                   | - |   |
|                                   | 4.2                           | -                                  | -                                   | -                                   | - |   |
|                                   | 4.3                           | ++                                 | -                                   | -                                   | - |   |
|                                   | 4.6                           | +                                  | +                                   | +                                   | + |   |
|                                   | 4.7                           | +                                  | +                                   | -                                   | - |   |
| OVA oral 500mg                    | 5.5*                          | IgM+                               | +                                   | +•                                  | - | + |
| MBP oral 500mg                    | 7.5*                          |                                    | +                                   | -•                                  | - | - |
| MBP nasal 50mg                    | 8.5*                          |                                    | +                                   | +                                   | + | - |
|                                   | 8.6*                          | IgM+                               | +                                   | -                                   | + | + |

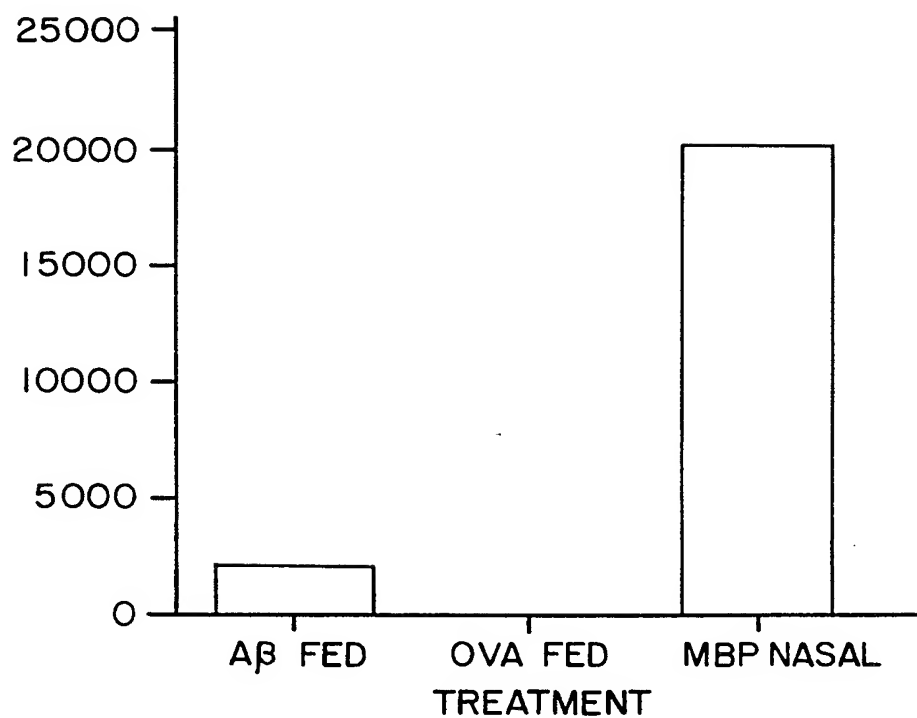
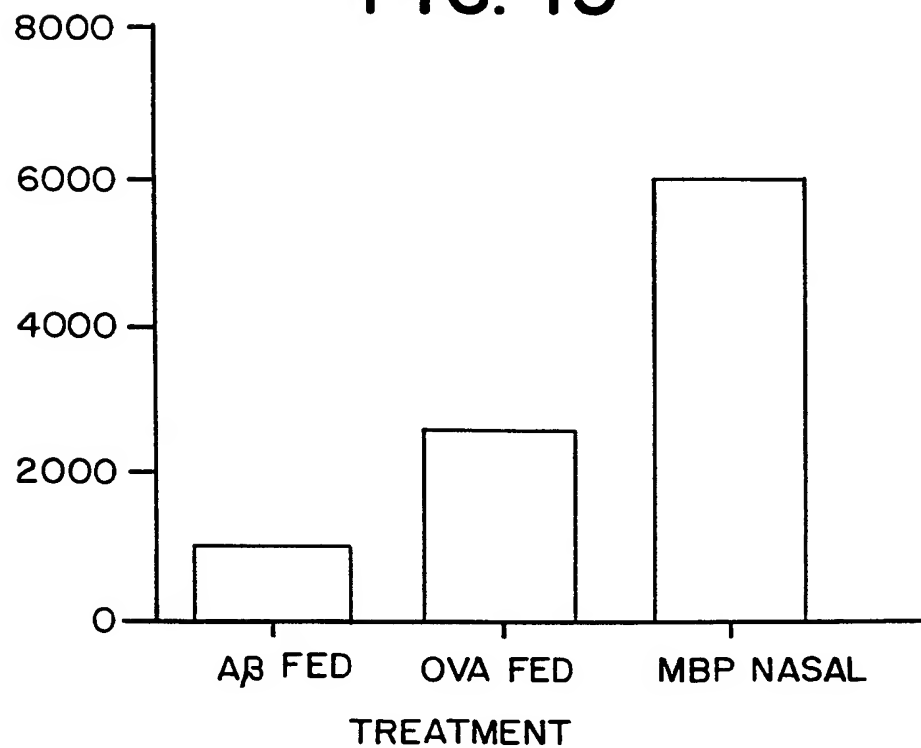
- negative

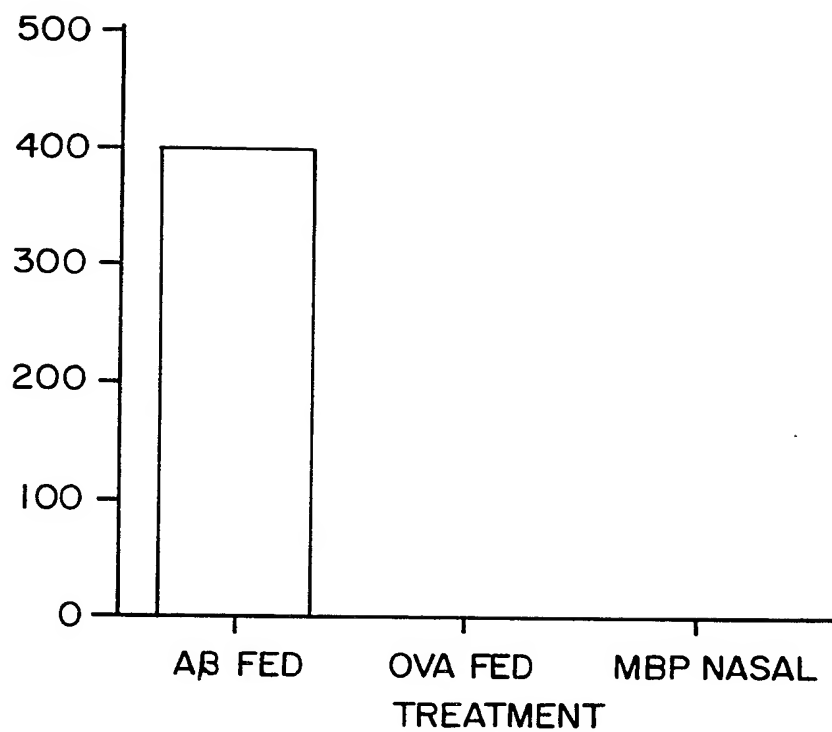
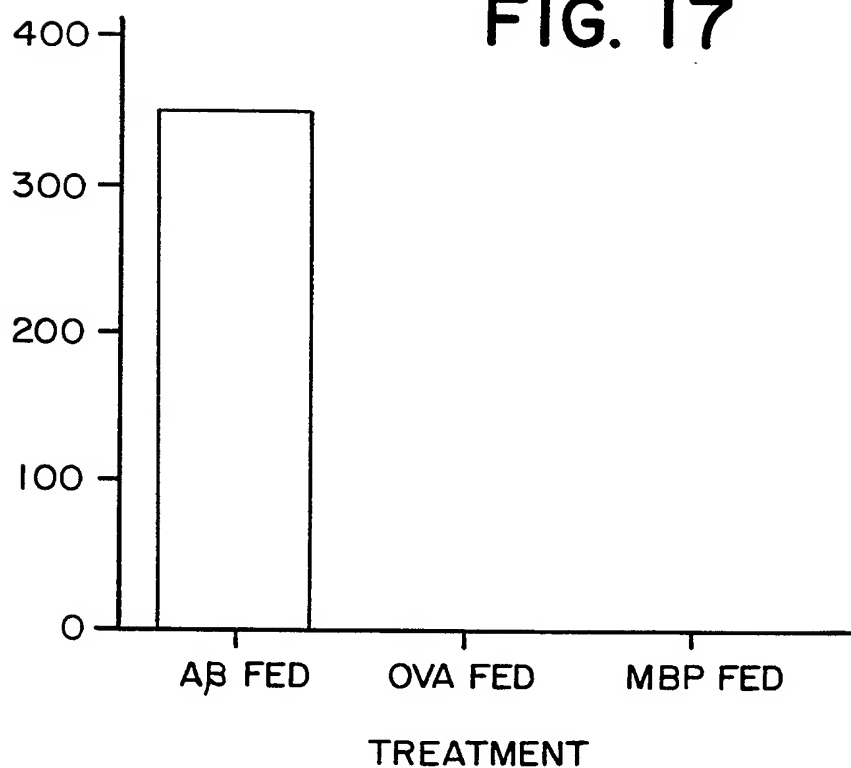
+ positive: sub-population of plaques stained or weak staining of numerous plaques

++ numerous plaques stained with strong immunoreactivity

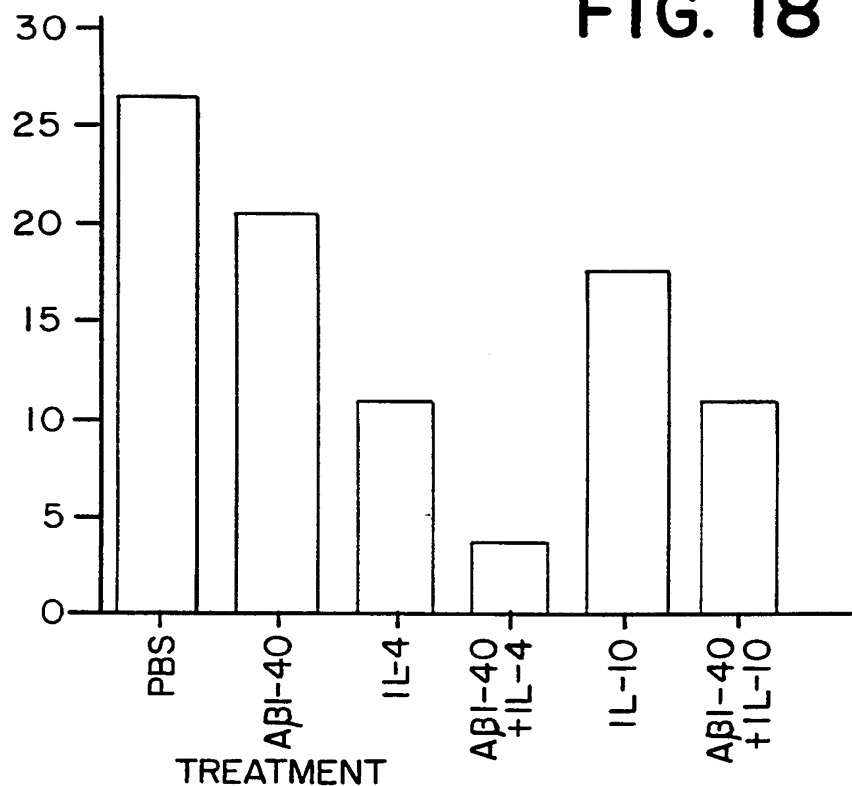
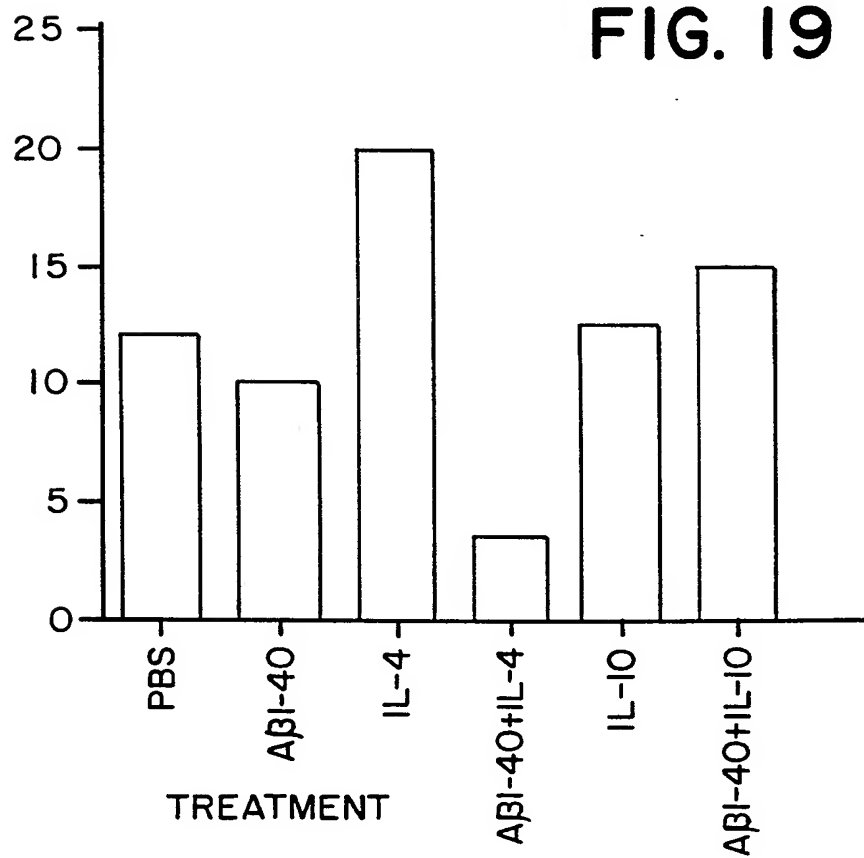
\* immunized with Ab(1-40) at 12 months, waited 10 days, then sacrificed

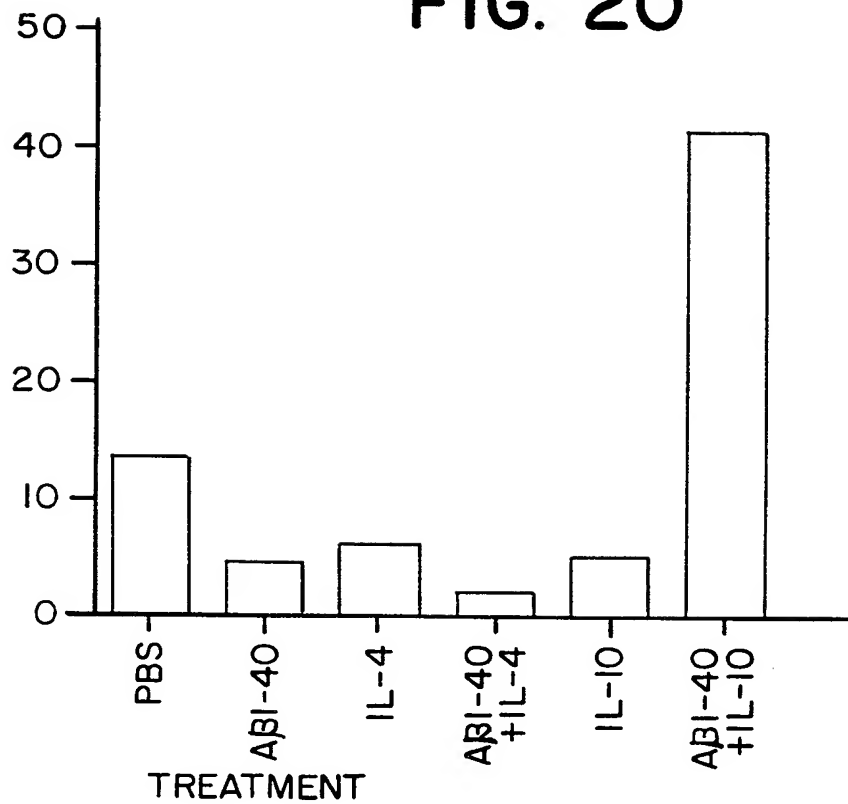
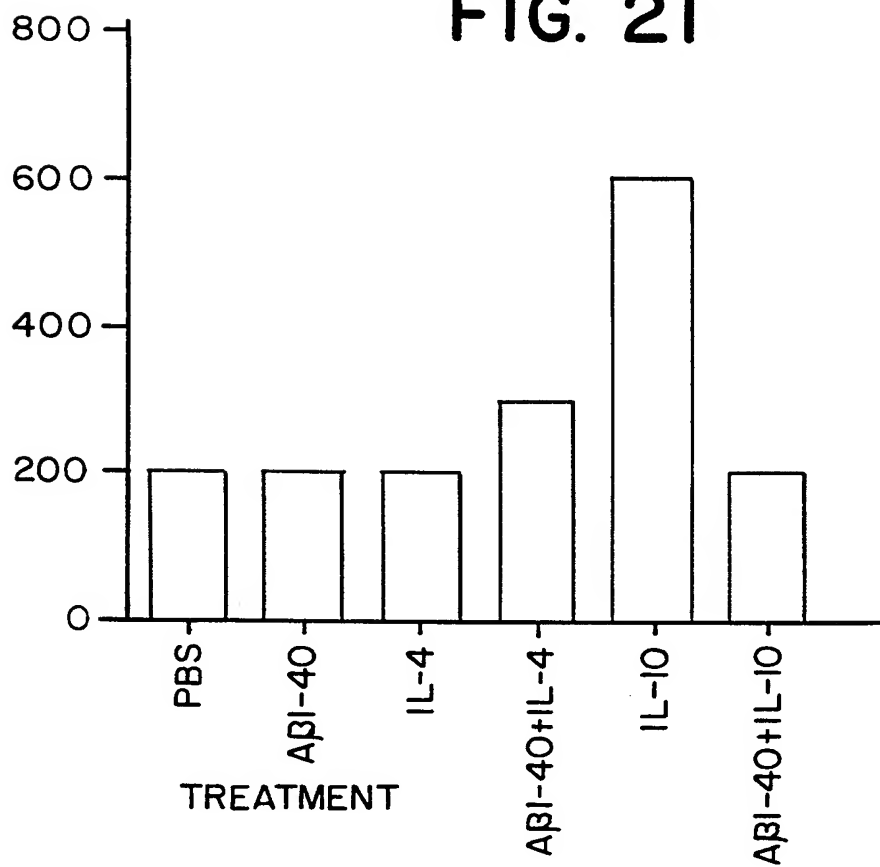
**FIG. 12****FIG. 13**

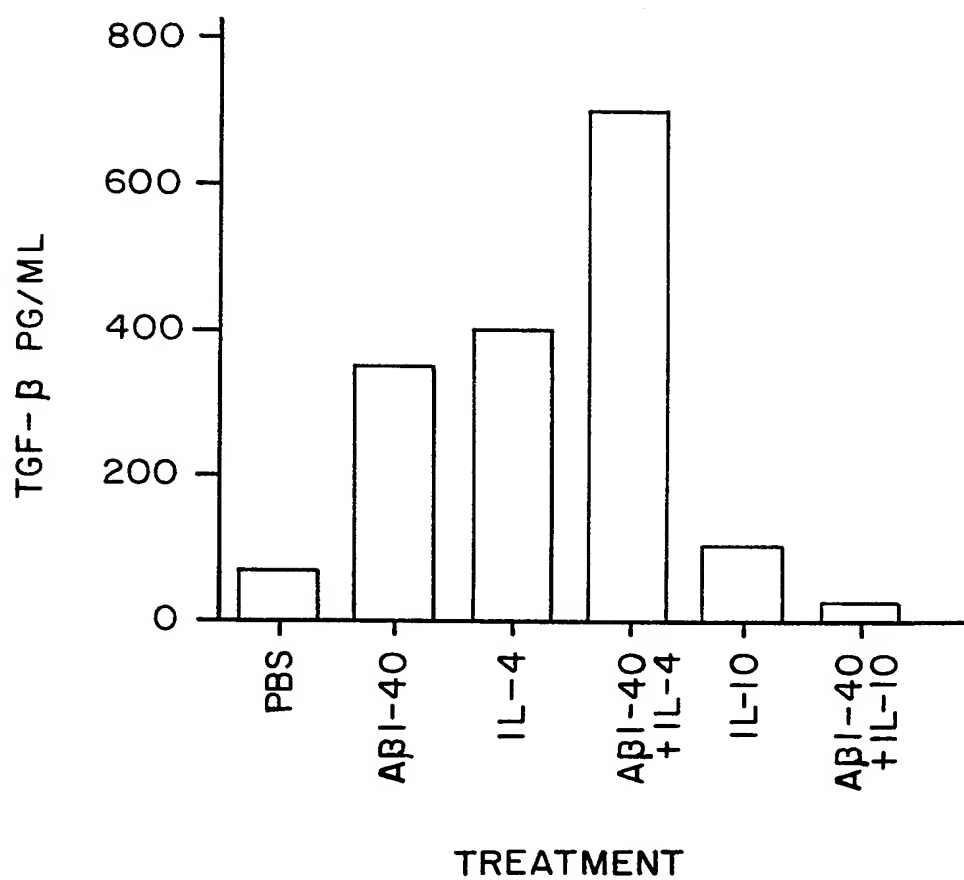
**FIG. 14****FIG. 15**

**FIG. 16****FIG. 17**



**FIG. 18****FIG. 19**

**FIG. 20****FIG. 21**

**FIG. 22**

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/25694

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 38/17

US CL :514/12, 885

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 885

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS and DIALOG (files 5, 155, 351, 357, 358) search terms: Alzheimer's, amyloid, mucosal, oral tolerance, inflammation, cytokine, nasal

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                             | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A         | WO 96/39176 A1 (BRIGHAM & WOMEN'S HOSPITAL) 12 December 1996 (12.12.96), see entire document.                                                                                                                                                  | 1-25                  |
| A, P      | TANAKA et al. NC-1900, an active fragment analog of arginine vasopressin, improves learning and memory deficits induced by beta-amyloid protein in rats. European Journal of Pharmacology. 1998, Vol. 352, pages 135-142, especially abstract. | 1-25                  |
| A         | GOZES et al. Neuroprotective strategy for Alzheimer disease: Intranasal administration of a fatty neuropeptide. Proceedings of the National Academy of Sciences. January 1996, Vol. 93, pages 427-432, especially abstract.                    | 1-25                  |



Further documents are listed in the continuation of Box C.



See patent family annex.

|                                                                                                                                                                         |                                                                                                                                                                                                                                                  |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| * Special categories of cited documents:                                                                                                                                | "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention                                              |
| "A" document defining the general state of the art which is not considered to be of particular relevance                                                                | "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone                                                                     |
| "E" earlier document published on or after the international filing date                                                                                                | "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" document member of the same patent family                                                                                                                                                                                                    |
| "O" document referring to an oral disclosure, use, exhibition or other means                                                                                            |                                                                                                                                                                                                                                                  |
| "P" document published prior to the international filing date but later than the priority date claimed                                                                  |                                                                                                                                                                                                                                                  |

Date of the actual completion of the international search

05 FEBRUARY 1999

Date of mailing of the international search report

17 FEB 1999

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

  
 MARIANNE P. ALLEN

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/25694

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                             | Relevant to claim No. |
|-----------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A         | FINCH et al. Evolutionary Perspectives on Amyloid and Inflammatory Features of Alzheimer Disease. Neurobiology of Aging. 1996, Vol. 17, No. 5, pages 809-815, see entire document.                                                                                                                             | 1-25                  |
| A         | TRIEB et al. Is Alzheimer beta amyloid precursor protein (APP) an autoantigen? Peptides corresponding to parts of the APP sequence stimulate T lymphocytes in normals, but not in patients with Alzheimer's disease. Immunobiology. 1994, Vol. 191, No. 2-3, page 114-115, abstract C.37, see entire document. | 1-25                  |

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>(51) International Patent Classification <sup>6</sup> :</b><br><b>C07K 14/435, A61K 39/00</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | <b>A1</b> | <b>(11) International Publication Number:</b> <b>WO 99/58564</b><br><b>(43) International Publication Date:</b> 18 November 1999 (18.11.99)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
| <b>(21) International Application Number:</b> PCT/NO99/00141<br><b>(22) International Filing Date:</b> 30 April 1999 (30.04.99)<br><b>(30) Priority Data:</b><br>19982098      8 May 1998 (08.05.98)      NO<br><b>(71) Applicant (for all designated States except US):</b> NORSK HYDRO ASA [NO/NO]; N-0240 Oslo (NO).<br><b>(72) Inventors; and</b><br><b>(75) Inventors/Applicants (for US only):</b> GAUDERNACK, Gustav [NO/NO]; Anthon Walles vei 17 A, N-1300 Sandvika (NO). ERIKSEN, Jon, Amund [NO/NO]; Bjørntvedt gt. 37, N-3916 Porsgrunn (NO). MØLLER, Mona [NO/NO]; Skrukkerødtoppen 8, N-3925 Porsgrunn (NO).<br><b>(74) Agent:</b> LILLEGRAVEN, Rita; Norsk Hydro ASA, N-0240 Oslo (NO). |           | <b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).<br><br><b>Published</b><br><i>With international search report.</i><br><i>With amended claims.</i><br><br><b>Date of publication of the amended claims:</b><br>27 January 2000 (27.01.00) |
| <b>(54) Title:</b> FRAMESHIFT MUTANTS OF BETA-AMYLOID PRECURSOR PROTEIN AND UBIQUITIN-B AND THEIR USE                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
| <b>(57) Abstract</b><br><br>Frameshift Mutants $\beta$ -Amyloid precursor peptides and mutant ubiquitin-B associated with Alzheimer's disease and Down syndrome eliciting T cellular immunity for use in compositions for the treatment and/or prophylaxis of Alzheimer's disease and/or Down syndrome.                                                                                                                                                                                                                                                                                                                                                                                                |           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

|    |                          |    |                                          |    |                                              |    |                          |
|----|--------------------------|----|------------------------------------------|----|----------------------------------------------|----|--------------------------|
| AL | Albania                  | ES | Spain                                    | LS | Lesotho                                      | SI | Slovenia                 |
| AM | Armenia                  | FI | Finland                                  | LT | Lithuania                                    | SK | Slovakia                 |
| AT | Austria                  | FR | France                                   | LU | Luxembourg                                   | SN | Senegal                  |
| AU | Australia                | GA | Gabon                                    | LV | Latvia                                       | SZ | Swaziland                |
| AZ | Azerbaijan               | GB | United Kingdom                           | MC | Monaco                                       | TD | Chad                     |
| BA | Bosnia and Herzegovina   | GE | Georgia                                  | MD | Republic of Moldova                          | TG | Togo                     |
| BB | Barbados                 | GH | Ghana                                    | MG | Madagascar                                   | TJ | Tajikistan               |
| BE | Belgium                  | GN | Guinea                                   | MK | The former Yugoslav<br>Republic of Macedonia | TM | Turkmenistan             |
| BF | Burkina Faso             | GR | Greece                                   |    |                                              | TR | Turkey                   |
| BG | Bulgaria                 | HU | Hungary                                  | ML | Mali                                         | TT | Trinidad and Tobago      |
| BJ | Benin                    | IE | Ireland                                  | MN | Mongolia                                     | UA | Ukraine                  |
| BR | Brazil                   | IL | Israel                                   | MR | Mauritania                                   | UG | Uganda                   |
| BY | Belarus                  | IS | Iceland                                  | MW | Malawi                                       | US | United States of America |
| CA | Canada                   | IT | Italy                                    | MX | Mexico                                       | UZ | Uzbekistan               |
| CF | Central African Republic | JP | Japan                                    | NE | Niger                                        | VN | Viet Nam                 |
| CG | Congo                    | KE | Kenya                                    | NL | Netherlands                                  | YU | Yugoslavia               |
| CH | Switzerland              | KG | Kyrgyzstan                               | NO | Norway                                       | ZW | Zimbabwe                 |
| CI | Côte d'Ivoire            | KP | Democratic People's<br>Republic of Korea | NZ | New Zealand                                  |    |                          |
| CM | Cameroon                 |    |                                          | PL | Poland                                       |    |                          |
| CN | China                    | KR | Republic of Korea                        | PT | Portugal                                     |    |                          |
| CU | Cuba                     | KZ | Kazakistan                               | RO | Romania                                      |    |                          |
| CZ | Czech Republic           | LC | Saint Lucia                              | RU | Russian Federation                           |    |                          |
| DE | Germany                  | LI | Liechtenstein                            | SD | Sudan                                        |    |                          |
| DK | Denmark                  | LK | Sri Lanka                                | SE | Sweden                                       |    |                          |
| EE | Estonia                  | LR | Liberia                                  | SG | Singapore                                    |    |                          |

## AMENDED CLAIMS

[received by the International Bureau on 1<sup>st</sup> December 1999 (01.12.99);  
original claims 1-25 replaced by amended claims 1-26 (5 pages)]

1. A peptide characterised in that it
- 5 a) is at least 8 amino acids long and is a fragment of a mutant  $\beta$ APP and/or Ubi-B protein arising from a frameshift mutation associated with Alzheimer's disease and/or Down syndrome;
- 10 and
- b) consists of at least one amino acid of the mutant part of the mutant  $\beta$ APP and/or Ubi-B protein;
- 15 and
- c) comprises 0-10 amino acids corresponding to the carboxyl terminus of the normal part of the protein sequence preceding the amino terminus of the mutant sequence and may
- 20 further extend to the carboxyl terminus of the mutant part of the protein as determined by a new stop codon generated by the relevant frameshift mutation;
- and
- 25 d) induces, either in its full length or after processing by antigen presenting cells, T cell responses.
2. A peptide according to claim 1 characterised in that it
- 30 contain 8-25 amino acids.
3. A peptide according to claim 1 characterised in that it contain 9-20 amino acids.



4. A peptide according to claim 1 characterised in that it contain 9-16 amino acids.

5 5. A peptide according to claim 1 characterised in that it contain 8-12 amino acids.

6. A peptide according to claim 1 characterised in that it contain 20-25 amino acids.

10 7. A peptide according to claim 1 characterised in that it contains 9 amino acids.

8. A peptide according to claim 1 characterised in that it contains 12 amino acids.

15 9. A peptide according to claim 1 characterised in that it contains 13 amino acids.

20 10. A peptide according to claim 1 characterised in that it is selected from a group of peptides having the following sequence identity numbers:  
seq id no. 1 - seq id no. 10 or a fragment of any of these.

25

30

35

11. A peptide for use in treatment of Alzheimer's disease or Down's syndrome,  
said peptide characterised in that it

5 a) is at least 8 amino acids long and is a fragment of a mutant  $\beta$ APP and/or Ubi-B protein arising from a frameshift mutation associated with Alzheimer's disease and/or Down syndrome;

10 and

b) consists of at least one amino acid of the mutant part of the mutant  $\beta$ APP and/or Ubi-B protein;

15 and

c) comprises 0-10 amino acids corresponding to the carboxyl terminus of the normal part of the protein sequence preceding the amino terminus of the mutant sequence and may  
20 further extend to the carboxyl terminus of the mutant part of the protein as determined by a new stop codon generated by the relevant frameshift mutation;

and

25

d) induces, either in its full length or after processing by antigen presenting cells, T cell responses.

30 12. A pharmaceutical composition comprising a peptide according to any of the above claims and a pharmaceutically acceptable carrier or diluent.

13. A vaccine for Alzheimer's disease comprising a peptide according to any of the claims 1-10 and a pharmaceutically acceptable carrier or diluent.

5 14. Use of a peptide according to any of the claims 1-10 for the preparation of a pharmaceutical composition for treatment or prophylaxis of Alzheimer's disease or treatment of Down syndrome.

10 15. Method for vaccination of a person disposed for or afflicted with Alzheimer's disease, consisting of administering at least one peptide according to the claims 1-10, one or more times, in an amount sufficient for induction of specific T-cell immunity to mutant  $\beta$ APP and/or  
15 mutant Ubi-B peptides associated with Alzheimer's disease and/or Down syndrome.

16. Method according to claim 15 wherein the amount of the peptides is in the range of 1 microgram (1  $\mu$ g) to 1 gram  
20 (1g) and preferentially in the range of 1 microgram (1  $\mu$ g) to 1 milligram (1 mg) for each administration.

17. Method for treatment of a patient afflicted with Alzheimer's disease or Down syndrome, by stimulating *in*  
25 *vivo* or *ex vivo* with peptides according to the claims 1-10.

18. Method according to claim 17 wherein the amount of the peptides used is in the range of 1 microgram (1  $\mu$ g) to 1 gram (1g) and preferentially in the range of 1 microgram (1  
30  $\mu$ g) to 1 milligram (1 mg) for each administration.

19. An isolated DNA sequence comprising a DNA sequence or variants thereof encoding a frameshift mutant peptide according to claim 1.

35

20. An isolated DNA sequence according to claim 19 encoding peptides comprising seq. id. no: 1-10 or variants thereof.

5 21. Use of a DNA sequence according to any of the claims 19-20 for the preparation of a pharmaceutical composition for treatment or prophylaxis of Alzheimer's disease or treatment of Down syndrome.

10 22. Method for treatment of a person disposed for or afflicted with Alzheimer's disease or afflicted with Down syndrome, by stimulating *in vivo* or *ex vivo* with DNA sequences according to the claims 19-20.

15 23. A plasmid or virus vector comprising DNA sequences of claim 18 encoding a frameshift mutant  $\beta$ APP peptide and/or Ubi-B peptide associated with Alzheimer's disease or Down syndrome.

20 24. A vector according to claim 23 wherein the vector is *E.Coli* plasmid, a *Listeria* vector and recombinant viral vectors. Recombinant viral vectors include, but are not limited to orthopox virus, canary virus, capripox virus, suipox virus, vaccinia, baculovirus, human adenovirus, SV40 or bovine papilloma virus.

25 25. Use of a plasmid or virus vector according to claim 23 for the preparation of a pharmaceutical composition for treatment or prophylaxis of Alzheimer's disease or treatment of Down syndrome.

30 26. Method for treatment of a person disposed for or afflicted with Alzheimer's disease or afflicted with Down syndrome, by stimulating *in vivo* or *ex vivo* with plasmids or virus vectors according to claim 23.

35